Analysis of B cell selection in autoimmune diseases

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STELLINGEN

B-cell selection in autoimmune diseases is different to that seen in classical antigen-driven selection. – This thesis

Acquisition of N-glycosylation motifs on the variable regions of B cell receptors may confer an altered B cell selection in autoimmune diseases through binding to lectins of the innate immune system. – This thesis

There are clear differences in the molecular characteristics of IgG transcripts derived from parotid glands of pSS patients versus non-pSS controls. – This thesis

B-cell depletion with rituximab fails to reset the core characteristics of immunoglobulin gene repertoires in salivary glands of treated pSS patients. – This thesis

Ig-producing cells that persist after rituximab treatment in salivary glands of pSS patients could contribute to the disease relapse observed after treatment. – This thesis

The grind of manuscript submission and review is unfair, with only reviewers allowed anonymity. Anonymity should be enforced for authors too, until publication. That should take care of crony politics and petty vengeance among the ostensibly intellectual elite.

Students can be a boon to inter-disciplinary research because, being unencumbered by expertise, they fearlessly lead (or carry) their supervisors into unfamiliar territories. - Irun R. Cohen, "Immune System Computation and the Immunological Homunculus", 2006

A uni-cultural experience leads to pride and prejudice; a multi-cultural adventure teaches tolerance and respect.

Truth is so obscure in these times, and falsehood so established, that, unless we love the truth, we cannot know it.- Blaise Pascal, 1623-1662; French mathematician, physicist, inventor, writer and Catholic philosopher.

Wise men speak because they have something to say; Fools speak because they have to say something. –Plato, ancient greek philosopher

A thoroughbred horse is not dishonoured by a poor saddle – an Arabic saying

The man who trims himself to suit everybody will soon whittle himself away. - Charles M. Schwab, 1862-1939, American steel magnate

Success is simple: Do what's right, the right way, at the right time. - Arnold H. Glasgow, American psychologist and author

Research presented in this thesis was generously supported by:
REUMAFONDS
GRONINGEN UNIVERSITY INSTITUTE FOR DRUG EXPLORATION (GUIDE)
JAN KORNELIUS DE COCK STICHTING

The printing of this thesis was supported by
REUMAFONDS
NATIONALE VERENIGING SJÖGRENPATIËNTEN
GRONINGEN UNIVERSITY INSTITUTE FOR DRUG EXPLORATION (GUIDE)
ROCHE NETHERLANDS
UNIVERSITY OF GRONINGEN (RuG)

ISBN: 978-90-367-5779-9

Lay-out and printing: Off Page, www.offpage.nl

Cover design and layout by Mr. Yahya Hautamäki, Al-Haqq Agency, Muscat, Sultanate of Oman; www.alhaqqagency.com

Cover interpretation: Autoimmune rheumatic diseases, such as Sjogren's syndrome, systemic lupus erythematosus and rheumatoid arthritis are more prevalent in women than men (Oliver et al; 2009). The shearing of the rose symbolizes this. The droplets of water on the rose petals symbolize the temporary clinical relief that current therapies in Western medicine are able to provide for patients suffering from autoimmune rheumatic diseases. The search for a complete cure however, continues.....

cmb.

RIJKSUNIVERSITEIT GRONINGEN

ANALYSIS OF B CELL SELECTION IN AUTOIMMUNE DISEASES

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
aan de Rijksuniversiteit Groningen
op gezag van de
Rector Magnificus, dr. E. Sterken,
in het openbaar te verdedigen op
maandag 22 oktober 2012
om 12:45 uur

door

Nishath Hamza
geboren op 18 november 1977
te Kizhakkambalam, India

Promotores	Prof. dr. N.A. Bos Prof. dr. C.G.M. Kallenberg Prof. dr. F.G.M. Kroese
Beoordelingscommissie	Prof. dr. L.F.M.H. de Leij Prof. dr. E. Vellenga Prof. dr. C.J.M. van Noessel

Dedicated to my husband, parents and darling sons,
Muhammad, Adam and Dawud

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INTRODUCTION

Autoimmune diseases are described as conditions where the immune system apparently turns against self-tissues and organs. They are generally classified into two types: Systemic and Localized autoimmune diseases. Systemic Autoimmune diseases are those that affect multiple organs such as systemic lupus erythematosus (SLE), rheumatoid artritis (RA) and Sjogren's syndrome (SS). They are usually characterized by the production of autoantibodies that recognize a diverse array of cytoplasmic and nuclear antigens. Localized autoimmune diseases, on the other hand, are organ-specific and often display autoantibodies that bind tissue-specific antigens.

B cells were initially thought to be minor players that acted more as the effector arms of the autoimmune process, as was evident from the excessive production of (auto)antibodies in patients with autoimmune disorders. However, the remarkable efficacy of therapeutic B cell depletion strategies indicated that B cells may play a major role in the very pathogenesis of autoimmune disorders. Moreover, B cells can modulate and amplify immune responses by mechanisms that do not necessarily involve antibodies (1) and increasing knowledge of different B cell functions have also added weight to the possibility that besides perpetuating the autoimmune process, B cells may actually cause autoimmune disease. Hence, any study of B cells in autoimmune disorders warrants a discussion of the many roles that B cells normally play within immune responses

B cells and antigen presentation

Besides antibody production, B cells also participate in antigen presentation and costimulation. Experiments on B cell-depleted mice clearly showed that B cells share the duty of antigen presentation with DCs. B cells seem to be participate in CD4+ T cell activation alongwith DCs during immune responses to low dose antigens (2). At the same time, B-cell-depleted mice are able to mount an immune response to a high dose of antigen, indicating that B cells are not indispensable for antigen presentation (3). However, when B-cell-depleted mice were exposed to autoantigens, reduced antigen-specific CD4+ T-cell activation was observed (4). All of this clearly suggest that B cells may be major antigen presenters that provide the trigger for autoimmune disease.

Role of B cells in lymphoid tissue organization and neogenesis

B cells are also known to regulate lymphoid tissue organization and neogenesis. In mice, the absence of B cells during development results in significant abnormalities within the immune system that is characterized by a significant decrease in the number and diversity of thymocytes, defects within spleen DC and T-cell populations (5), an absence of Peyer's patch organogenesis and follicular DC networks alongwith decreased chemokine expression (6, 7). On the other side of the coin, B cells are also implicated in formation of ectopic lymphoid follicles in diseased organs or tissues in autoimmune disorders. For example in primary SS (pSS), these ectopic lymphoid structures are characterised by periductal clusters of T and B lymphocytes, development of high endothelial venules and differentiation of follicular dendritic cells (FDC) networks (8, 9). Evidence from pSS patients and SS animal models demonstrated that the formation and maintenance of such lymphoid follicles is strongly associated with the expression lymphotoxins and lymphoid chemokines CXCL13, CCL19, CCL21 and CXCL12 in diseased salivary glands (8). Another example of the prominent role B cells play in the formation of lymphoid follicles was demonstrated by an elaborate study of ectopic lymphoid neogenesis and in-situ autoantibody production in the pancreatic tissue of autoimmune diabetic mice (10) which provided further clarity on the sequence of events possibly catalyzed by B cells in autoimmune diseases.

B cells regulate T cell differentiation

Initial studies on B cell-deficient mice indicated that the absence of B cells adversely affected both CD4+ T cell and CD8+ T cell responses (11-13). This has been confirmed by B cell depletion studies in humans, at least wth regards to CD4+ T cells. B cell depletion therapy in SLE patients resulted in decreased numbers of CD4+ T cells expressing the costimulatory molecule, CD40-ligand (14). This confirms that B cells have a major role to play in T cell differentiation. This is probably why B cell depletion therapy is also seen to be clinically effective in treating autoimmune diseases that are understood to be T cell-mediated (15).

Immunomodulating functions of B cells

B cells respond to Toll-like receptor (TLR) ligands and present antigen. In addition, B cells may be subdivided into regulatory and effector B cell subsets based on the functions of the cytokines they produce (16). Regulatory B cells secrete IL-10 or TGFβ-1, while effector B cells produce IFNγ, IL-12 and TNFα (Be-1 cells) or IL-2, IL-4, TNFα, IL-6 (Be-2 cells) depending on whether the antigenic response induced is type-1 or type-2, respectively.

Regulatory B cells are particularly significant in the context of B cell hyperactivation disorders. For example, IL-10 producing B cells have an ameliorating effect experimental models of colitis (17), EAE (18) and collagen-induced arthritis (19). The role of IL-10-producing B regulatory cells in down-regulating immune responses became evident in infections (20, 21) and autoimmunity (22-25). Similarly, the regulatory function of TGFβ-1-producing B cells has also garnered much attention (26, 27). These facts indicate that the effector and regulatory arms of B cell immunomodulation work independently to influence T cell-mediated immune responses.

ROLE OF B CELLS IN AUTOIMMUNTIY

Under healthy conditions, B cells follow tightly regulated developmental and differentiation pathways intercepted by many check points to prevent the distraction of a homeostatic immune response into autoimmunity or uncontolled B cell hyperactivity. B cells develop from stem cells within the bone marrow, through a series of precursor stages during which they rearrange their variable immunoglobulin (Ig) genes to generate a wide range of B cell receptors (BCRs) with unique antigen-binding specificities. Immature transitional B cells expressing surface BCRs emigrate from the bone marrow into the peripheral blood and then mature into naïve B cells. Although the site of maturation is not known in humans, in mice, this process occurs in the spleen. When mature naïve B cells encounter antigen and are able to solicit Dendritic cell (DC) and T-cell help within the follicles of secondary lymphoid organs, they undergo germinal center (GC) reactions leading to somatic hypermutation (SHM) of their rearranged Ig gene combinations, proliferation and Ig heavy-chain class-switch recombination. Thus, the GC is a unique microenvironment poised for the production of memory B cells or Ig-secreting plasma cells from naïve B cells which are responsible for the maintenance of serum antibody levels. These complex molecular processes are unique to B cells and ensure the production of antibodies with a variety of antigen-binding specificities and effector functions (by different isotypes). Antibodies function both as a natural barrier to infection and as a humoral component of adaptive responses to pathogens.

Autoimmune diseases are primarily characterized by the excessive production of antibodies that can bind self-antigens and are accompanied by a cascade of pathological events that result

in the immune system being harnessed to destroy specific cells of the body. It is apparent that at some point in the development of autoimmunity, the usual checkpoints aimed at eliminating self-reactive B cells have failed. The etiology behind autoimmune diseases in general, remains a mystery. Although several theories abound, some of them contradictory to each other (28, 29), the one overriding fact is that autoimmune disorders are generally accompanied by autoantibodies with the ability to bind self-antigens. Although, it cannot be stated with certainity that all autoantibodies are pathogenic, their association with autoimmune disease initiation and progression makes B cells a frontline in exploring the mechanisms underlying autoimmune mechanisms. With each B cell having a unique BCR, it is evident that the study of gene sequences encoding for the BCRs in autoimmune disease could divulge critical information as to how B cells in autoimmune disorders and in particular auto-reactive B cell populations, differ from the B cells in healthy controls.

THE B CELL REPERTOIRE IN AUTOIMMUNE DISEASES

The B cell repertoire is derived from an array of gene rearrangements that can potentially form immunoglobulins (Ig) of almost unlimited specificities. The genes of the variable (V), diversity (D; in Ig heavy chain only) and the joining (J) regions have the potential to come together in numerous permuted combinations to form unique B cell receptor configurations. This process of somatic recombination is choreographed in a sequential manner and interspersed with various checkpoints that are essential to the generation of a vastly diversified yet functionally optimal antibody repertoire that has the ability to respond effectively to non-self antigenic stimuli.

In humans, the process of V(D)J recombination begins with the rearrangement of genes within the heavy chain (H) locus in the progenitor B cells (pro-B) located in specialized niches within the haematopoietic fetal liver and bone marrow. First, the DH-JH gene segments join, followed by the VH gene segment combining to form a unique VH-DH-JH rearrangement which allows the pro-B cell to become a precursor B cell (pre-B) that produces mu heavy chains. It is now evident that almost all pro-B cells have DH to JH rearranged at both alleles, indicating their prompt availability for VH to DHJH-rearrangements (30).

IgL chain rearrangement normally starts off at the kappa locus. If the Vkappa-Jkappa combination is unproductive, then the IgL chain rearrangement machinery moves on to the lambda locus. Either way, the expression of a productively rearranged light chain along with a functional mu heavy chain enables the presentation of a mature BCR on the surface of the naïve B cell and is a crucial signal for the survival and differentiation of the B cell.

Although potential V(D)J recombinations required to produce the diverse immune repertoire seem to occur at random, there are several stringent checkpoints in place within the early B cell developmental pathway that ensure the removal of unwanted recombination products and the generation of optimal BCRs. B cells with unproductive V(D)J rearrangements which largely arise due to the randomness of the recombination machinery, do not survive unless they are rescued by further functional rearrangements on the same or different allele for the heavy chain and a different light chain locus (lambda) for the light chain. A second checkpoint ensures the selection of only those functional heavy chains that are able to transiently pair with single light chains to form a pre-BCR. Finally, only functional light chains that can pair with heavy chains to form a mature BCR, are selected.

Abnormalities in Ig gene diversification

VH replacement

Even when a complete BCR is finally expressed on the B cell membrane, a new kind of checkpoint kicks in, which focuses on the antigen-specificity of the BCR and ensures that B cells with self-reactive BCR specificities are either eliminated or undergo receptor editing of the light chain to rid itself of self-reactivity. This secondary rearrangement is particularly easy at the kappa and lambda loci due to the gene organizations where an upstream VL can join to a downstream JL as they are both flanked by complementary RSS sites. A previous unproductive or self-reactive VL-JL rearrangement can easily be replaced by a new VL-JL recombination (31).

Such a form of secondary rearrangement is mechanistically difficult to envision at the heavy chain locus, since the initial DH-JH joining deletes out the intervening DH genes along with the 12bp RSS required for recombination with the 23bp RSS of a new upstream VH. Despite this, growing evidence that a form of secondary rearrangement known as VH replacement may occur at the heavy chain locus, came from comparisons of functional and non-functional VH-DH-JH rearrangements in mouse B cells (32, 33). It was suggested that VH replacement may be mediated through the use of cryptic RSS (cRSS) sequences located within the third framework region of the VH germline gene segments. In mice and humans, almost all functional VH germline genes contain cRSS motifs alongside a heptamer (TACTGTG) without a clearly definable nonamer partner (34). The occurrence of VH replacement in acute lymphoblastic leukemias (ALL) of pre-B cell phenotype had been indicated (35, 36) previously. But it was the study of a tetraploid childhood acute lymphocytic leukemia cell line EU12 (37) that provided mechanistic evidence for the contribution of VH replacement to the IgH gene diversification process in humans.

The concept of VH replacement gained significance in autoimmunity when knock-in mice with a self-reactive (anti-DNA) VH-DH-JH transgene engineered into the JH locus were found to have the inserted anti-DNA VH gene replaced by upstream VH, DH, or VH-DH gene fragments through recombination with a cRSS heptamer embedded at the 3'-end of the anti-DNA VH transgene (38, 39). The VH replacement in these mice apparently sought to delete the anti-DNA specificity at the pre-B to immature B transitional stages in the bone marrow (39), presumably after encounter with self-antigen (31). Other studies in mice have implicated VH replacement in the purging of self-reactive BCR specificities (40), in repertoire diversification for mounting protective anti-viral Ig response (41) and in the production of monoclonal antibodies against different immunogens (42).

Receptor editing

Receptor editing is the process by which the autoreactivity of B cells is modified through a secondary rearrangement at the light chain locus. There is some evidence to indicate that receptor editing may be defective in certain autoimmune disorders. However, the results have also been quite contradictory. For example, in SLE, studies have shown that there is a preferential use of proximal Vκ genes and the infrequent utilization of downstream Jκ segments in anti-DNA Abs indicating decreased receptor editing (43). In contrast, other studies showed that usage of Vλ genes of the most Jλ-proximal cluster was decreased while that of the most distal cluster was increased in untreated SLE patients compared to normal controls, suggesting an increased occurrence of receptor editing (44)(45). In patients with SS, over-representation of Jλ2/3 and a decreased frequency of Jλ7 were noted in rearrangements in peripheral B cells, suggesting decreased receptor editing (45). SS and SLE patients also exhibit significant

differences between their productive and nonproductive repertoire such that SLE patients showed decreased productive rearrangements for Vλ4 and Vλ5 and increased for Vλ6. The SS repertoire, on the other hand, demonstrated productive rearrangements increased for Vλ7 and decreased for Vλ10 (46).

SCOPE OF THIS THESIS

While it is evident that B cells could be major contributors to the disease pathology in many autoimmune diseases, such as Sjogren's syndrome, it is still unclear as to which factors allow B cells to evade the normal checkpoints for autoimmunity and transgress the boundaries of tolerance. Moreover, although B-cell depletion with Rituximab is effective in treating many autoimmune diseases such as primary Sjögren's syndrome (pSS), patients always suffer a disease relapse and the factors contributing to this relapse are unknown (47-49). This thesis aimed at analyzing the immunoglobulin variable heavy chain repertoire to uncover possible mechanisms used by B cells to survive, proliferate and persist in autoimmune disorders and particularly in pSS.

METHODOLOGIES FOR IMMUNOGLOBULIN GENE ANALYSES

Experimental techniques

Immunoglobulin gene rearrangements are analyzed using polymerase chain reactions (PCR) with V region-specific primers in combination with either J region-specific primers when analyzing DNA or with constant region primers when analyzing RNA/cDNA. The PCR products obtained are inserted into plasmid vectors and amplified in copy numbers by cloning using bacterial cells. The bacterial cells are cultured on agar plates and single bacterial colonies are picked for further culturing, since each colony presumably stems from an individual bacterial cell with a single plasmid-PCR insert combination. The plasmid-insert DNA is then extracted out from the bacterial cells and sequenced using plasmid-specific primers upstream and/or downstream of the PCR insert.

Analyzing the Ig sequences at the RNA level has the advantage of providing focused information on only those B cells where the Ig DNA locus recombination is expressed as mRNA and possibly transcribed into protein. Also, the final spliced and processed mRNA transcript has both the variable and constant regions of the Ig molecule close enough to be detected within the same PCR, thereby allowing the characterization of BCR-specific isotype usage.

The downside of using mRNA for analyzing Ig sequences is that activated B cells and plasma cells are known to produce upto 1000-fold more RNA than resting B cells. Thus, analysis of the total mRNA pool may be heavily biased towards the Ig gene repertoire of these hyperactive cells. Theoretically though, this mRNA bias may be overcome by studying definite sorted B cell populations. But, it may also lead to underestimating the auto-antibody producing plasma cell populations in autoimmune disorders since definitive plasma cell markers are still a matter of debate.

The Ig-mRNA bias towards the message from plasma cells may also be overcome by analyzing the Ig repertoire at the DNA level. However, the DNA sequences obtained would also include those uninformative sequences which are not transcribed into RNA and do not contribute to the Ig repetoire. Moreover, these sequences do not provide information on isotypic constant

regions because the variable region and constant region genes are too far apart on the Ig genomic locus to be ampilified within the same PCR. The knowledge of isotype expression with variable gene usage may be important within the context of clinical correlations (50).

Regardless of whether DNA or RNA is used, an additional bias is also introduced by the PCR-plasmid cloning approach, where the number of sequences analyzed (which is actually the number of bacterial cells picked during cloning) may vary depending on transformation efficiency, which in turn is affected by factors such as amount of PCR product, ligation efficiency, etc. This makes it difficult to come to quantitative conclusions about Ig gene usages and repertoire features in general, as Ig mRNA sequences dervied from cells with higher copy number transcripts such as plasma cells or DNA sequences that are amplified more efficiently due to better primer binding, stand the chance of being cloned more often.

To this end, the recent approach of using parallel deep sequencing strategies for the analysis of Ig mRNA transcripts can eliminate most of the above-mentioned biases as the entire Ig transcript pool is sequenced. In this way, the number of times an Ig sequence is detected is a near accurate quantitation of the Ig transcript production and the parallel amplification of a genomic DNA locus would also normalize for B cell numbers. However, analyzing the massive amounts of sequence data generated after deep sequencing remains a daunting task requiring extensive bioinformatic support and computational capacity.

Computational immunology

Ig sequence data analysis has been greatly aided by the availability of web-based open-source Ig gene databases such as IMGT (51), JoinSolver (52) and Kabat (53). The query tools within these databases provide a variety of features such as V-(D)-J gene identification, functionality (protein coding) prediction, CDR3 amino acid sequence, hypermutation hotspots, replacement and silent mtation statistics, etc.

In recent years, use of these online query tools were incorporated into different mathematical algorithms designed to predict the type and extent of selection pressures that shape the Ig repertoire on the basis of mutation patterns. In this context, a defining moment was the realization that although unproductive sequences do not contribute to the Ig pool, they are crucial to differentiating the somatic hypermutations occurring in response to antigenic exposure, from the random mutations acquired during recombination events and cell divsions (54). This is because unproductive sequences do not code for a functional protein (BCR) and mutations acquired by such sequences are probably uninfluenced by ongoing antigen selection.

The prediction of selection pressures on Ig sequences is crucial as they aid in determining the role of antigen-driven selection in disorders such as autoimmunity and B cell lympohomas. These methods are essentially based on comparing the observed frequency of replacement (non-synonymous) or R mutations to their expected frequency under the null hypothesis of no selection where mutations, even if they occur, are silent or S (synonumous). Higher R/S frequencies indicate positive antigen selection, while decreased frequencies indicate 'negative selection' or a decreased propensity to accrue R mutations that optimize antigen binding and the statistical significance for these tests are determined. Logically, one can expect negative selection (less R mutations) in the framework regions (FWRs), as these regions form the structural backbone of the BCR and positive selection in the complementarity determining regions (CDRs), which are responsible for antigen binding in response to antigenic stimulation.

The first statistical testing algorithm for evaluating the effect of antigen selection on B cells was created by Chang and Casali (55) and was later modified to form the Multinomial test by

Lossos et al (56). In 2008 however, the Focused-z test (previously known as Focused Binomial test) was developed (57, 58) which as its name suggests, only focuses on the region (FWR or CDR) being analyzed and does not allow statistical cross-talk between regions. For example, a low number of R mutations in the FWR may falsely indicate a relative increase in the R mutations (positive selection) within the CDR if they are not analyzed separately. As a result, the focused test is a more robust approach in contrast to the Chang and Casali method and the Multinomial test, where the number of R mutations are compared between CDR and FWR. Moreover, the Focused test also takes into account the effect of microsequence specificity (59) and base substitution bias (60) in the occurrence of somatic hypermutations, thereby greatly reducing the likelihood for false positives in predicting antigen selection.

A considerable improvement on the Focused test is the recently published computational method known as the Bayesian estimation of Antigen-driven SELectIoN (BASELINe) (61). BASELINe provides a more quantitative and visual method to analyze selection. It incorporates statistical algorithms designed to predict the type and extent of selection pressures which shape the Ig repertoire on the basis of mutation patterns. Affinity maturation is a process by which B cells accumulate somatic mutations within their immunoglobulin genes after which they are selected based on their affinity to antigens. The distribution of replacement versus silent (R/S) mutations, in both CDRs and FWRs is counted separately and compared against the expected frequency under the hypothesis of no selection as defined by germline genes. This strategy is different from the Focused-z test, in that the Focused-z test only facilitated the detection of sequences showing statistically significant selection pressures, but did not offer a way to quantify the large number of sequences where selection pressures were not statistically significant and merely indicated trends (57, 58). BASELINe on the other hand allows the cummulative quantification of selection pressures within a repertoire, as well as a tandem comparison of selection pressures between different experimental groups (61).

In addition to the analyses for selection pressures, the translated (amino acid) Ig sequences can also be analyzed for their predicted protein characteristics such as hydrophobicity/ hydrophilicity, charge, N-glycosylation sites, etc., some of which are shown in this thesis to differ in autoimmune diseases when compared to normal controls [Chapters 4 and 5].

THESIS OUTLINE

In this chapter, we demonstrated the importance of studying the role of B cells in autoimmune diseases. Given that B cell have a multi-faceted and mutli-layered contributions within different types of immune responses, it is likely that abnormalities in B cell repertoires may possibly give rise to the pathology seen in autoimmune diseases.

In Chapter 2, we present a review of the B cell populations and sub-population in pSS and also discuss the effect of B cell depletion therapy on the B cell repertoire in pSS.

In Chapter 3, we presented evidence for the persistence of immunoglobulin-producing cells in parotid salivary glands of primary sjögren's syndrome patients after B-cell depletion therapy with Rituximab.

In Chapter 4, we proposed, for the first time, the existence of altered selective pressures on IgG-producing cells in parotid salivary glands of pSS patients.

In Chapter 5, we extrapolated our previous strategy for analyzing altered B cell selection in pSS (Chapter 4) to the immunoglobulin heavy chain (IGHV) gene repertoires of other autoimmune diseases. We showed, for the first time, the existence of novel selective pressures

in the form of acquired N-glycosylation motifs in IGHV regions of IgG-producing cells from autoimmune diseases. This study also showed that the prevalence of acquired N-glycosylation sites in autoimmune diseases was significantly greater than in IgG sequences from normal or non-autoimmune and antigen-specific repertoires.

In Chapter 6, we delved further into the concept of altered selective pressures in autoimmune diseases and introduced novel insights regarding the selection pressures shaping the autoimmune repertoire. We also used the observations from this study to challenge the popular belief that B cell hyperactivation in autoimmune diseases is due to (auto)antigen-driven stimulation. In this study, we concluded that the selection pressures on IgG-expressing B cells in autoimmune diseases are more similar to superantigen-based B cell selection than classical antigen-driven B cell selection.

In Chapter 7, we combined our observations from previous chapters to introduce a novel hypothetical model of altered B cell selection in autoimmune diseases. We speculate that our study may tentatively provide a first evidence for the much discussed, but undetermined link between B cell superantigen infections and autoimmunity.

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B CELL POPULATIONS AND SUB-POPULATIONS IN SJÖGREN'S SYNDROME

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Accepted; in press – La Press Medicale, 2012

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ABSTRACT

Sjögren's Syndrome (SS) is a chronic inflammatory disorder affecting exocrine glands, in particular the lacrimal and salivary glands. The disease can be primary (pSS) or secondary to other systemic autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus and others. The systemic autoimmune character of pSS is also apparent from the occurrence of (non-organ specific) autoantibodies in this disease. Histopathologically, glandular involvement is characterized by focal accumulation of lymphocytes, particularly around epithelial ducts, with, sometimes, germinal center-like structures. The infiltrates largely consist of T-cells, with a preponderance of CD4-positive T-cells. As a result, the pathology in SS was primarily attributed to T cells. However, a break with the fixation on the role of T cells in pSS came when therapeutic B cell depletion strategies proved remarkably efficacious in this disease, thereby indicating a major role for B cells in the immunopathogenesis of pSS.

In this regard, a closer look at the composition of B-cells and B-cell subpopulations, both in the peripheral blood and in target tissues, is worthwhile. In this review we discuss current data on B-cells in pSS. B-cell depletion offers a unique possibility to study the recurrence of (pathogenic) B-cells and their characteristics in pSS patients treated with rituximab. Data on B-cell subpopulations in the peripheral blood and B-cell repertoire in the target tissues following rituximab treatment are discussed as well. We also address their state of activation, repertoire, and relation to B-cell activating factor (BAFF).

INTRODUCTION

Sjogren's syndrome (SS) is a rheumatic autoimmune disorder that primarily affects glandular tissues that produce moisturizing secretions such as the salivary and lacrimal glands. It is known as primary Sjogren's syndrome (pSS) when it occurs on its own and as secondary Sjogren's syndrome when it occurs in tandem with other systemic autoimmune diseases such as systemic lupus erythrematosus (SLE) or rheumatoid arthritis (1). Extraglandular involvement is frequent, indicating that pSS is a systemic autoimmune disease. There is a strong female preponderance with a female to male ratio of 9 to 1, and a high prevalence of up to 3% in people above the age of 50 years (2).

For many years, the immune pathology in SS, as well as many other autoimmune diseases, was largely attributed to T cells as they were observed to inflitrate in large numbers within affected organs or glands. Experiments with autoimmune animal models also showed that T cells could transfer autoimmune diseases (3). Diseases such as rheumatoid arthritis and insulin-dependent diabetes mellitus also showed strong statistical associations with certain MHC class II alleles, which was suggestive of a T cell-dependent process. However, a break with the fixation on the role of T cells in autoimmune disorders came when anti-T cell therapy failed to produce desirable clinical results in certain systemic autoimmune diseases (4).

B cells on the other hand, were initially thought to be minor players that acted more as part of the effector arm of the autoimmune process, as was evident from the excessive production of (auto)antibodies in patients with autoimmune disorders. In pSS, the classical symptoms of hypergammaglobulinemia and increased production of autoantibodies such as anti-Ro/SSA and anti-La/SSB were evidence of B cell hyperactivation. Moreover, a quarter of pSS patients also exhibit ectopic development of B cell proliferations which resemble germinal centres (GC) in the target tissues and approximately 5-10% of them have the probability of progressing to MALT lymphoma, a B cell malignancy (5, 6).

These observations suggested a more primary role for B-cells in the immunopathogenesis of pSS. Consquently, B-cell depletion with the chimeric monoclonal antibody Rituximab was attempted in patients with pSS. The initial data was promising and showed restoration of glandular function in early pSS with positive effects as well on extraglandular manifestations and constitutive symptoms like fatigue (7). In view of these findings, a closer look at the composition of B-cells and B-cell subpopulations, both in the peripheral blood and in target tissues, is worthwhile. In this review we will discuss current data on B-cells in pSS. B-cell depletion offers a unique possibility to study the recurrence of B-cells and their characteristics in pSS patients treated with rituximab (RTX). Data on B-cell subpopulations in the peripheral blood and the B-cell repertoire in the target tissues following RTX treatment will be discussed as well. We will also address their state of activation and their response to the B-cell activating factor, BAFF.

B cell populations in pSS patients

In peripheral blood

Many studies, including those from our group, have given evidence for disturbances in the distribution of peripheral blood B cell subsets in pSS patients. In comparison to healthy controls, the peripheral blood from pSS patients exhibit a significant decrease in frequencies and numbers of CD27+ memory B cells and an increase in those of both naïve and memory CD27− B cells (8-12). There are two possible explanations for the decrease in CD27+ memory B-cells in

the peripheral blood of pSS patients. One could be the migration of CD27+ memory B-cells in patients into inflamed salivary glands due to the increased expression of the chemokines CXCL12 and CXCL13 in these glands (13). The other possibility is that shedding of CD27 from the cell surface resulting in CD27− memory B-cells (10) may account for the reduced detection of circulating CD27+ memory B-cells in pSS patients.

The latter possibility seems quite plausible since CD27- memory B-cells were also reported in healthy individuals. These B cells were mostly of the IgG isotype and were somewhat less mutated than their CD27+ counterparts (14). Strikingly, increased frequencies of CD27− memory B-cells that were class-switched were reported in the blood of SLE patients, and were positively correlated with higher disease activity and increased levels of disease-specific autoantibodies (15). Our own group reported a significant negative correlation between frequencies and numbers of circulating CD27− B-cells and salivary gland function in pSS patients (16). Given the fact that IgM−IgD+CD27− B-cells from healthy individuals were shown to bind to autoantigens, this raises the possibility that the subset of CD27- B cells may be the precursors of autoantibody secreting plasma cells in autoimmune diseases (16, 17). Analyzing the presence of these subsets within affected glands of pSS patients may shed more light on the role of these B cells in the autoimmmune disease pathology. In addition to this, our group also reported a significant increase in the percentages and absolute numbers of circulating transitional B cells, as well as a significant increase in the percentages (but not in the numbers) of naive CD27−CD38low B cells in pSS patients compared to healthy individuals (8).

Another study that explored the presence of long-lived plasma cells (PCs) in pSS, reported a significantly higher percentage of PCs in the peripheral blood of pSS patients with lymphocytic infiltration focus scores ≥ 2 in their salivary glands compared to those pSS patients with a focus score ≤ 1 (18). The authors also revealed a two-fold higher proportion of CD19− PCs compared to CD19+ PCs in pSS patients than in normal controls. The expression of CD27 in both these subsets of PCs from pSS patients was observed to be significantly lower than in normal controls (18). Interestingly, the lack of CD19 and CD27 from the surface of PCs is reportedly associated with the susceptibility to malignancies (19, 20). Since reduced CD19 expression on long-lived PCs within the bone marrow is correlated with increased lifespan of these cells (21), these cells could be the precursors for long-lived PCs in pSS patients (18). However, it remains to be elucidated whether these CD19- long-lived PCs play a role in either persistent autoantibody production or the increased susceptibility to B cell malignancies observed in pSS patients, or both.

In salivary glands

Studies on B cell subsets present in the salivary glands of pSS patients are few and not very definitive. The lymphocytic infiltrates seen in the salivary glands of pSS patients are more akin to B cells clusters or aggregates rather than typical germinal centers (GCs) owing to their lack of typical GC-markers such as CD10 and CD38 and the expression of activation-induced cytidine deaminase which is required in GC reactions for somatic hypermutation and class-switching (22). These B cells lacked expression of CD38, which is usually present on transitional T1-type B cells that emigrate from the bone marrow into the circulation (23). This indicated the presence of a more mature subset of transitional B cells, termed T2 B cells, which were CD19+IgD+CD38-IgM+CD21+CD23+ and present within pSS salivary glands(24) (22). However, another study on lower lip salivary gland (LSG) biopsies reported that in contrast to the widely dispersed presence of B cells expressing CD38+, CD79a+ and CD5+ to a minor degree, and lacking CD20, CD21 and CD27 in LSGs from healthy controls, LSGs from pSS patients showed CD20+ B cells and

CD27+/CD38+ B cells, with the CD20+ B cells being concentrated in perivascular regions that did not overlap with focal infiltrates filled with the CD27+/CD38+ B cell subset (25). A more recent study also detected significant numbers of CD138+, non-proliferating, Bcl-2 expressing plasma cells in the salivary glands of pSS patients with high focus scores (26).

Our group [Hamza et al, submitted] and others (27-29) showed increased clonal expansions of immunoglobulin-producing cells (B cells and plasma cells) in the salivary glands of pSS patients compared to non-pSS control patients. In our study, some of these clones were composed of mixed IgA and IgG isotypes within the parotid salivary glands from pSS patients. This suggested the presence of localized class switching within the inflamed glands of pSS patients (30). These observations suggest the existence of local B-cell hyperactivation and proliferation in the affected tissues.

In Sjögren's syndrome, the presence of anti-Ro (SS-A) and anti-La (SS-B) autoantibodies against RNA-protein complexes are an important diagnostic criterium (31). Studies have shown that pSS patients with relatively high levels of anti-Ro/SSA and anti-La/SSB autoantibodies in their sera also presented with anti-Ro/SSA and anti-La/SSB producing cells in their labial glands, particularly along the periphery of germinal center-like structures and interstitial spaces within the labial glands (6, 32). Moreover, pSS patients who presented with germinal center-like microenvironments within their salivary glands, showed increased local production of anti-Ro/SSA and anti-La/SSB autoantibodies and apopotic activity within these microenvironments, thereby indicating the significance of a highly localized interaction between the target glandular tissue and immune cells in pSS autoimmune pathology (6). Some SS patients had detectable titres of anti-Ro and anti-La autoantibodies in plasma, yet no autoantibody-producing cells were found in their peripheral blood (33). This suggests that (auto)antibody production probably occurrs in tissues such as the bone marrow or in secondary lymphoid organs such as lymph nodes and tonsils. In diseases such as pSS, the sites of inflammation within salivary glands may also be used for this purpose. The (auto)antibody-specific memory B cells may have an increased tendency to migrate to these inflammed sites due to the increased expression of chemoattracts such as CXCL12 and IL-6 (discussed later) within salivary glands of pSS patients (18) where the inflammatory microenvironment may contribute to their transformation to (auto)antibody-producing plasma cells.

Abnormalities of B cell immunomodulation in pSS

It has been suggested that CXCL13 and CXCL12 overexpression in the inflamed glands of pSS patients could play an active role in the recruitment of B cells as infiltrating cells (34). Peripheral blood B cells from patients with primary SS show significantly higher gene expression of surface CXCR4 in contrast to healthy individuals and this was especially evident with respect to CD27- B cells. However, transmigration assays based on the interaction between CXCR4 and its cognate ligand CXCL12 showed that the migratory potential of peripheral CXCR4+ CD27– B cells from pSS patients was not different from that of healthy controls (13). This may be due to the fact that significantly higher frequencies of CD27– naive B cells from pSS patients expressed the mRNA for the inhibitory regulator of G protein signaling 13 (RGS13), which is known to inhibit the migrational response of CXCR4-transfected Chinese hamster ovary cells toward CXCL12 and CXCL13 in vitro (13, 35).

Activated CD27+ B cells from primary SS patients showed significantly reduced migratory responses to the high expression of CXCL12 and CXCL13 in pSS glands when compared with those from healthy controls. However, analysis of peripheral blood revealed a moderately diminished

frequency of CXCR5+ CD27+ memory B cells in pSS patients compared to healthy controls. This was surprising because the CXCL13–CXCR5 pairing has been shown to be involved in the homing of B cells into lymphoid follicles, as well as in the development of organized lymphoid follicles (13). Given the low CXCR5 expression on CD27+ B cells that are left in the pSS peripheral blood, the question arises as to which factors facilitate the migration of CXCR5+ CD27+ memory B cells into the inflammed salivary glands of pSS patients and the formation of ectopic lymphoid tissue within them (36, 37). This is a significant point because CD27+ memory B cells were shown to be reduced in the peripheral blood and accumulated in the inflammed salivary glands. The vast majority of these infiltrating CD27+ memory B cells coexpressed CXCR5 and CXCR4, while the lower frequencies of peripheral CD27+ memory B cells were accompanied by a reduction in CXCR4+ and CXCR5+ B cells in pSS patients. Thus, coexpression of both CXCL12 and CXCL13 may attract the peripheral CXCR4+CXCR5+CD27+ memory B cells into the inflamed glands, where they may reside and proliferate. This suggests that a process of selective migration leaves memory B cells with lower migratory capacity remaining in the blood while those that can home into the inflammed glands may be retained within the glands through as yet undefined survival signals (13).

A recent study observed that CXCL12 and interleukin (IL)-6 survival factors were highly expressed in pSS salivary gland epithelium and within focal mononuclear infiltrating cells. Adipocytes present in the salivary glands were also proven to be an important source of CXCL12 (26). Strikingly, plasma cells were detected in close proximity to CXCL12 and IL-6 expressing cells, suggesting that CXCL12 and IL-6 may be vital for plasma cell survival (26).

Another group of cytokines that play important roles in B-cell survival, differentiation and proliferation are the cytokine B-cell activating factor (BAFF) and the proliferation-inducing ligand (APRIL). Both BAFF and APRIL can bind to either of the receptors BCMA (B cell maturation Ag) and TACI (transmembrane activator and calcium modulator and cyclophilin ligand activator), but only BAFF binds another receptor, BAFF-R or BR3 (38). BAFF-transgenic mice develop a condition that has certain similarities with the human pSS condition (39). In pSS patients, both BAFF and APRIL levels are increased (40). In BAFF-transgenic mice, autoreactive marginal zone B-cell clonal populations that apparently proliferated in the spleen, were also found within salivary glands, indicating that cells deriving from the splenic marginal zone population may be the precursors for autoreactive cells in human pSS (16, 39).

In pSS patients BAFF was shown to be produced not only by epithelial cells and T cells but also by B cells (41). An interesting observation though, was the fact that the BAFF receptor BR-3 was present on most B cells within the salivary glands of pSS patients while TACI and, to a lesser degree, BCMA were observed on transitional B lymphocytes. This was suggestive of a form of autocrine feedback mechanism for B cell activation and proliferation (41). Furthermore, only the epithelial cell-bound BAFF extended the survival of normal B cells while the secreted BAFF did not do so (41). This is significant in view of the fact that studies on self-reactive B cells in BAFF transgenic mice have demonstrated that BAFF overexpression can promote the survival of autoreactive B cells that are usually deleted during later stages of maturation and also faciltates their migration into niches from which they are normally excluded (42). These observations also highlight the possible involvement of the glandular tissue (epithelial cells) within salivary glands in perpetuating the pSS disease process.

Elevated serum BAFF and APRIL levels in pSS patients were positively correlated to serum gammaglobulins, IgG, presence of anti-SSA or anti-SSB autoantibodies and focus score (39, 43, 44). Moreover, BAFF expression within inflamed salivary glands was associated with the

presence of germinal center-like structures (45), while elevated BAFF levels in the serum of pSS patients correlated with the increased number of peripheral CD27–CD38++IgD+ B-cells (46).

As a result of all of these observations, anti-BAFF therapy is now being considered a serious option in the treatment of pSS. Belimumab, a monoclonal antibody to BAFF, has already shown significant benefits for patients with SLE (47), which is a disease that is comparable to pSS in its presentation of higher BAFF and APRIL levels in the sera of patients. To our knowledge, clinical trials targeting BAFF in pSS patients are still ongoing.

A pertinent development in the study of BAFF expression was the detection of multiple alternatively spliced transcripts for BAFF. An alternatively spliced mRNA, in which exon 3 is absent was shown in mice to negatively regulate BAFF by forming heterotrimers with the full-length form transcript. However, the translated form of this BAFF transcript has not been detected (48). Another BAFF RNA variant which lacked a 114 base pair region within exon 4 of the BAFF gene was found to encode for a protein that could interact with DNA and perform as a transcription factor for the full-length BAFF transcript (24). It remains to be seen if the differential expression of the former and/or the latter BAFF variant(s) in pSS patients compared to healthy controls may be a factor driving the excessive BAFF expression seen in pSS and other autoimmune diseases.

A recent study using co-culture experiments of human salivary gland (HSG) cell line cells and tonsilar B lymphocytes, showed that direct HSG cell-B cell contacts were able to induce apoptosis in epithelial cells (49). This B cell-mediated apoptosis required the translocation of protein kinase C delta (PKC δ) into the nucleus of epithelial cells, which then resulted in histone H2B phosphorylation on serine 14 and poly (ADP-ribose) polymerase cleavage (49). This is particularly noteworthy since the treatment of B cells with BAFF is reported to prevent nuclear accumulation of PKCδ and enhance B cell survival (50).

Selection of the autoimmune immunoglobulin repertoire in pSS

Previous studies have shown that the immunoglobulin variable light chain repertoire within peripheral blood B cells exhibits a disordered selection in pSS patients compared to controls. The VL-λ genes 2A2, 2B2, 2C and 7A together accounted for 56% of all functional Vλ joints (51), whereas in another study of peripheral blood from a single pSS patient, 43% of the VL- κ repertoire was represented by the Vκ genes L12, O12/O2 and B3 (52). A study on a parotid gland biopsy from the same patient revealed an increased usage of the rheumatoid factor and lymphoma-associated VκA27 gene (29%) compared with blood-derived B cells (8%) (53). Moreover, an increased frequency of B cells expressing the VκA27–Jκ2 rearrangement was observed within the parotid glands of this pSS patient. It remains to be seen whether this apparent bias in Vk gene usage is also seen in larger series of pSS patients. However, with regards to the immunoglobulin variable heavy chain gene usage, we (30) found no evidence for a biased usage of a particular Vh gene in pSS patients compared to non-pSS controls.

With regards to isotype usage, we noted significant differences in IgA and IgG subclass usage in the salivary glands of pSS patients compared to non-pSS controls. In parotid glands from pSS patients, among IgA transcripts, the expression of IgA1 was significantly higher than IgA2 (Figure 1), whereas among IgG transcripts, IgG1 was the dominant IgG subclass [Hamza et al, submitted]. In non-pSS controls on the other hand, the dominant IgA and IgG sublasses were IgA2 and IgG2, respectively. It is uncertain whether increased IgA1 expression could contibute to pSS disease pathology. However, the role of IgG1 could be significant since IgG1 is thought to be more effective at phagocytosis and complement-fixation than IgG2 (54). Hence, the relative

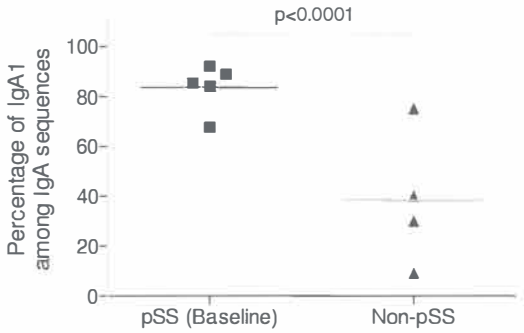

Figure 1: IgA1 expression is increased in pSS patients. The frequency of IgA1 subclass usage among IgA transcripts in parotid gland biopsies from pSS patients at baseline compared to that in non-pSS controls (p-value from Mann-Whitney test).

predominance of IgG1-expressing cells which possibly bind (auto)antigens within the diseased salivary glands of pSS patients, may attract a powerful complement-mediated detrimental immune response locally, leading to the pathology observed in the salivary glands of pSS patients [Hamza et al, submitted].

B cell depletion therapy in pSS

B-cell depletion therapy using the anti-CD20 monoclonal antibody Rituximab (RTX) resulted in clinical relief from disease symptoms in treated pSS patients and is characterized by a near complete depletion of B cells from the blood (8, 55). Although a significant therapeutic effect was observed with respect to various disease parameters such as saliva production, visual analog scale (VAS) scores for dryness (particularly in patients with recent disease onset), fatigue and extraglandular manifestations (56-60), the clinical relief experienced by these patients was temporary and the recurrence of disease symptoms coincided with the return of B cells within the peripheral circulation (8, 55).

Following RTX treatment, repopulation of the peripheral compartments was characterized by the appearance of CD19+ B-cells at 24 weeks which were phenotypically akin to CD27- $CD38^{high}$ transitional B-cells and whose numbers normalized partially or fully at 36–48 weeks after RTX (8, 61). This indicates the influx of newly-generated B cells from the bone marrow into the peripheral circulation of treated pSS patients. Among the memory B cell populations in the peripheral blood of pSS patients treated with RTX, approximately 70% of them belonged to an isotype-switched subset described by the markers CD19+ CD27+ IgM- IgD-. However, in our studies on concomittant parotid gland biopsies from the same pSS patients, we observed that the near complete depletion of B cells from the peripheral circulation noted at 12-16 weeks after RTX, was not mirrored by the parotid salivary glands and B cells were still present there (30, 59). This is in contrast to previously reported observations where a complete absence of B-cells was noted in the labial salivary glands biopsies of pSS patients for upto one year after RTX (61). The repopulation of B cells within these labial glands was mostly represented by memory and transitional B-cells.

We have previously reported the persistence of certain clonal populations of immunoglobulin (Ig)-producing cells within the parotid glands of pSS patients even after B cell depletion with RTX (30). One obvious reason for the survival of these Ig-producing cells could be the lack of CD20 expression on plasma cells, as is widely believed. However, there are reports of CD20+ plasma cells being present in tonsils even after RTX (62). We also

showed that Ig-producing clonal populations persisting after RTX were more mutated in the variable region of the immunoglobulin heavy chain (IGHV) genes than their clonally-related counterparts present before RTX (30). This is an indication that B cells surviving after RTX may have undergone proliferation.

All of the above observations point to a model of disease relapse after RTX therapy that may be seeded by persisting Ig-producing cells. The surviving B cells may be situated in restricted niches that enable them to evade depletion and proliferate with time. The existence of such niches with restrictive access to RTX was previously reported in murine systems, where B-cells in certain tissue sites such as the splenic marginal zone, germinal center and peritoneal cavities exhibit significant resistance to anti-CD20 depletion (63, 64). These RTX-resistant B cell niches were also observed in the GCs of lymph nodes from non-human primates (65) and in tonsils (66) and other lymphoid organs in humans (67).

The transient clinical relief from SS symptoms after RTX is probably due to the depletion of large numbers of B-cells and some CD20+ Ig-producing cells (62), which may lead to a reduction in effector B-cell functions, such as antigen presentation and cytokine production. Although no significant decrease in serum Ig levels was observed after RTX (58), lower levels of certain (auto)antibodies may contribute to clinical relief. At the same time, the underlying autoimmune mechanisms are probably maintained by long-lived plasma cells as has been indicated in studies in SLE where patients who expressed autoantibodies secreted by long-lived plasma cells, had a higher chance of experiencing early flares or disease relapse after B-cell depletion therapy than patients who did not express these autoantibodies (68-71).

Serum BAFF levels have been measured in pSS patients before and after rituximab therapy (72). Following rituximab therapy, serum BAFF levels increase significantly during periods of B cell depletion and BAFF levels return to baseline when B cell numbers return to normal values. The augmented BAFF levels following B cell depletion may contribute to the return of self-reactive B cells, as excessive BAFF has been shown to rescue self-reactive B cells from apoptosis (73). In our studies, we showed that although RTX treatment does result in the almost complete depletion of peripheral B cell populations, its effect on the B cell populations within diseased salivary glands is not so dramatic. Moreover, compared to baseline, we found no significant changes with respect to the relative usage of the immunoglobulin heavy chain gene repertoire or IgA and IgG isotype subclass usage in pSS patients after RTX treatment [Hamza et al; submitted]. Hence, the B cells that survive after RTX may ultimately contribute to the disease relapse observed in pSS patients who have undergone RTX therapy (30). This is a sound rationale for the development of therapeutic strategies that combine RTX with an anti-BAFF agent to prolong the therapeutic efficacy of RTX (74). Alternatively, designing therapies that target persisting Ig-producing cells (or plasma cells) could also synergistically increase the efficacy of B cell depletion therapy (68).

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5 PERSISTENCE OF IMMUNOGLOBULIN-PRODUCING CELLS IN PAROTID SALIVARY GLANDS OF PRIMARY SJÖGREN'S SYNDROME PATIENTS AFTER B-CELL DEPLETION THERAPY

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Ann Rheum Dis. 2012 May 21; in press.

ABSTRACT

Objectives: To assess the persistence of immunoglobulin-producing cell populations in the parotid salivary glands of primary Sjögren's syndrome (pSS) patients after B-cell depletion therapy with Rituximab.

Methods: Thirteen pSS and 4 control patients were included in this study. pSS patients were treated with Rituximab or placebo. Sequence analysis was carried out on IgA and IgG encoding transcripts extracted from parotid salivary gland biopsies, taken before, at 12-16 and at 36-52 weeks after treatment.

Results: At baseline, many clonally-related sequences were observed in pSS patients. The number of clonal expansions was significantly higher in pSS patients, compared to control patients. Clonal expansions were composed of IgA and/or IgG expressing cells. Rituximab did not significantly alter the degree of clonal expansions. Groups of clonally-related cells had members which were shared between biopsies taken before and after treatment. Mutation frequencies of immunoglobulin sequences from clonally-related cells in pSS patients were higher after treatment.

Conclusions: Rituximab treatment does not alter the characteristic features of increased clonal expansions observed in the parotid salivary glands of pSS patients. The presence of clonally-related immunoglobulin-producing cells before and after Rituximab treatment strongly suggests that immunoglobulin-producing cells persist in salivary glands of pSS patients despite B-cell depletion. The presence of mixed isotype expression within groups of clonally-related cells indicates local class-switching in salivary glands of pSS patients. We speculate that persistent immunoglobulin-producing cells may underlie disease relapse after treatment.

INTRODUCTION

Primary Sjögren's syndrome (pSS) is an autoimmune disease characterized by chronic inflammation of the salivary and lacrimal glands (1) and progressive dryness of mouth and eyes. The presence of B-cell infiltrates in the salivary glands of pSS patients, accompanied by disturbances in the relative proportions of peripheral B-cell subsets and high titers of circulating autoantibodies such as anti-Ro (SS-A) and anti-La (SS-B) antibodies in the blood, strongly implicate B-cells in the pathogenesis of pSS (2). This assumption was strengthened when therapeutic B-cell depletion strategies targeting CD20, a B-cell-specific transmembrane protein, resulted in relief from SS disease symptoms (3-7).

B-cell targeting therapies with anti-CD20 monoclonal antibodies, such as the chimeric antibody Rituximab (RTX) are now widely used to treat autoimmune disorders such as rheumatoid arthritis (RA), systemic lupus erythromatosus (SLE) and vasculitis (8). We and others have previously shown that RTX can also be successfully used for the treatment of patients with pSS (3, 6, 9, 10).

However, symptoms usually return 6-9 months after treatment (6, 10). Although, RTX treatment results in the almost complete absence of B-cells in the peripheral blood of pSS patients for 3-6 months after the initiation of the therapy (10-12), this is not mirrored by their parotid salivary glands, where B-cells remain, albeit in decreased numbers (7). Also, disease relapse seemingly coincides with the reappearance of peripheral B-cell subpopulations in the blood (9-11).

B-cells can be distinguished from each other on the basis of the unique rearrangements of their immunoglobulin variable heavy (IGHV) and light chain genes. B-cell clones (and plasma cells derived from them) share the same IGHV rearrangements. Previous studies have reported the presence of B-cell clones within salivary gland biopsies from pSS patients using immunoglobulin sequence analysis (13-15). In order to assess whether such B-cell clones possibly persist in salivary glands of pSS patients after RTX treatment, we analyzed immunoglobulin sequences obtained from repeated biopsies taken from the same parotid gland before and after RTX treatment. If clonally-related Ig-producing cells are observed before and after RTX treatment in pSS patients, this may strongly suggest a potential role for these persistent cells in the disease relapse occurring following B-cell depletion therapy with RTX.

METHODS AND MATERIALS:

Patients

Thirteen pSS-patients fulfilling the American-European criteria for pSS(16) (all females; mean age 40.5 years; range 18-65 years), with a disease duration of less than 5 years, were enrolled in this study after providing written informed consent. The study protocol was approved by the institutional review board of the University Medical Center Groningen. Patients included in this study had participated in previous pilot studies such as the open label Rituximab study (n=4) (6) or in the placebo controlled Rituximab study (n=9) (9).

Inclusion criteria for pSS-patients to participate in this study were: stimulated whole saliva secretion flow > 0.15 ml/min, presence of autoantibodies (IgM-rheumatoid factor $\geq$ 10 kIU/liter in combination with anti-SS-A and/or anti-SS-B autoantibodies), and a salivary parotid gland biopsy (obtained $\leq$ 12 months before inclusion) showing characteristic features for SS (17). Patients with a history of any malignancy (such as MALT lymphoma) or underlying cardiac, pulmonary, metabolic, renal or gastrointestinal conditions or with chronic or latent infectious diseases or immune deficiency, were excluded. Furthermore, patients who had been treated previously with

monoclonal antibodies were excluded. Treatment with prednisone and hydroxychloroquine had to be discontinued at least one month before baseline, and treatment with other disease-modifying anti-rheumatic drugs (DMARDs) at least 6 months before baseline.

Eleven patients (pSS-RTX patients) had been treated according to the following two schemes: a RTX infusion of 375 mg/m^2/week for 4 weeks (in the open label study; n=4) (6) or 2 i.v. infusions of 1000 mg RTX (in the placebo controlled trial; n=7) (9) on days 1 and 15. Two patients (pSS-placebo patients) had been treated with placebo infusions (9). To minimise side effects (infusion reactions, serum sickness), all patients, both RTX and placebo-treated had been pre-medicated with methylprednisolone (100 mg/i.v.), acetaminophen (1000 mg/p.o.), and clemastine (2 mg/i.v.) and received 60 mg of oral prednisone on days 1 and 2, 30 mg on days 3 and 4, and 15 mg on day 5 after each infusion. No corticosteroids or other DMARDs were allowed during follow-up.

As controls (all females; mean age51 years; range 45-57 years), 2 patients with sicca complaints not fulfilling the American-European criteria for pSS and 2 patients with malignancies (squamous cell carcinoma of the oral cavity) without involvement of the parotid glands were included.

An incisional biopsy of the parotid gland was obtained from the same gland before therapy (RTX or placebo) and 12-16 weeks later (17). Moreover, in 5 pSS-RTX patients, a third biopsy was taken at 36-52 weeks from baseline, at the time of recurrence of clinical symptoms. In control patients, a biopsy of the parotid gland was obtained either as part of the clinical diagnostic work-up for Sjögren's syndrome or as part of the neck dissection procedure in patients with malignancy during surgical intervention. Histopathological examination of the parotid glands of all 4 control patients revealed a normal histology of the glandular tissue.

Cloning and sequencing of immunoglobulin transcripts

From every biopsy, three batches of four serial tissue sections (5-7μm thick) were obtained from different parts of each biopsy. Total RNA was extracted from each batch. RNA was converted to cDNA and amplified using primers specific for the variable region of IGHV genes (18) in combination with constant region primers specific for the CH1 domains of the Cα (5'-GAATTC GAGTGGCTCCTGGGGGAAGA-3') or Cγ (5'-GAGTTCCACGACACCGTC AC-3') constant regions (19). For 4 pSS-RTX patients and the 4 control patients, we performed a multiplex PCR for all IGHV families. For the remaining 9 pSS-RTX treated patients, we focused our analysis on the largest IGHV family, i.e. IGHV3. PCR was performed for 35 cycles using a 60-second denaturizing step at 94^{0}C, 60-second annealing step at 60^{0}C and a 60-second extension step at 72^{0}C. The PCR products were gel-extracted and cloned into plasmid vectors using the Fermentas GeneJet Kit and Xli Blue® competent bacteria. A total of 72 (36 IgA + 36 IgG) plasmid-PCR product constructs were picked separately for each biopsy and submitted for sequencing. Only plasmids containing IGHV sequence-inserts were considered further for analysis.

The independent sampling of different parts of each biopsy ensured that 100% identical immunoglobulin sequences derived from separate tissue batches could be attributed to different cells. Identical immunoglobulin sequences within a single tissue batch could be derived from multiple transcripts of the same cell or from different cells. For this reason 100% identical sequences obtained from one tissue batch were counted as one.

Analysis of rearranged immunoglobulin genes

The nucleotide sequences of the variable region were compared with the international ImMunoGeneTics information system (IMGT) databases of human immunoglobulin germline

sequences (20, 21). Imunoglobulin sequences obtained from independent PCRs were assigned to clonally-related immunoglobulin-producing cells based on their similarity at the complementarity determining region (CDR) 3 and their shared IGHV gene usage (22). Shared mutations within the IGHV regions were also considered to be indicative for clonal relationships. Mutation frequencies of immunoglobulin sequences were computed with an in-house software tool (23).

Immunohistochemistry

Parotid glands were fixed in formaldehyde (4%), embedded in paraffin and sectioned. The sections were stained after deparaffinisation, pre-treatment with Ultra CC1 (Ventana Medical Systems, Inc, USA), antigen retrieval and endogenous peroxidase blocking using the Benchmark machine. Sections were immunohistochemically stained with polyclonal IgA (1:12000), polyclonal IgG (1:32000) and monoclonal CD79a, clone: JCB117 (dilution 1:100) antibodies. All antibodies used were from DAKO. The sections were then treated with peroxidase-labelled secondary antibody and visualized with the chromogen DAB (3,3' Diaminobenzidine) solution.

Statistical analysis

All statistical analyses were performed using GraphPad Prism software (version 3.0). Statistical comparisons of data from pSS patients and control patients were carried out using unpaired t-test test. Data from baseline and at different timepoints after treatment were analyzed using paired t-test.

RESULTS

A total of 1,314 immunoglobulin (803 IgA and 511 IgG) sequences were collected from patients with pSS (at baseline and after RTX treatment) and from control patients. Of these sequences 109 (8%) were designated as unproductive (non-protein coding) by the IMGT Quest bioinformatic tool. After filtering out unproductive sequences and 100% identical sequences from the same PCRs, the number of productive (coding for a functional protein) immunoglobulin sequences from different immunoglobulin-producing cells were 1,172 (1,084 from pSS patients and 88 from control patients) and only these sequences were used for subsequent analysis.

Increased clonal expansions in parotid salivary glands of pSS patients

Analysis of immunoglobulin sequences obtained from parotid salivary glands, from both pSS patients and control patients, clearly revealed the existence of sequences derived from clonally-related cells. In pSS patients, at baseline, on average >20% ± 1.85 of the sequences collected from a parotid gland biopsy belonged to clonally-related cells, whereas in control patients the average percentage (7% ± 2.37) was significantly lower (P=0.0027, unpaired t-test; Figure 1a). The number of groups of clonally-related cells per biopsy (normalized to 100 sequences/biopsy) from pSS patients at baseline was also significantly higher than compared to those from control patients (P=0.0022, unpaired t-test; Figure 1b) and the sizes (i.e. the number of clonally related sequences) of these clonal groups were significantly larger in pSS patient biopsies at baseline, than in control patients (P=0.0457, unpaired t-test; Figure 1c).

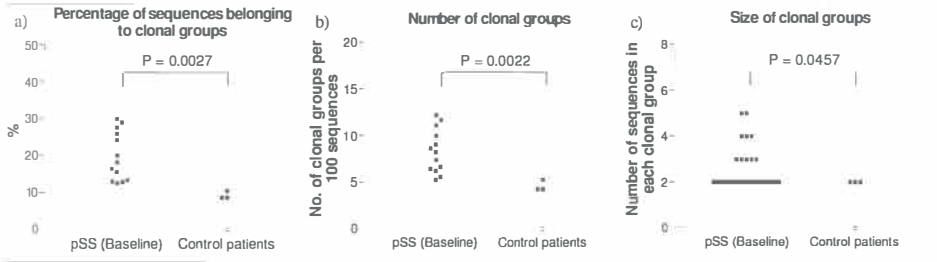


Figure 1: Immunoglobulin sequences from clonally-related cells detected in parotid gland biopsies from pSS patients and control patients. a) Percentage of immunoglobulin sequences belonging to clonally-related cells in biopsies from pSS patients at baseline compared to those from control patient biopsies. b) Number of groups of clonally-related cells per biopsy (normalized to 100 sequences/biopsy) from pSS patients at baseline compared to those from control patient biopsies. c) Size of clonal groups from pSS patients at baseline compared to those from control patient biopsies

Clonally-related cells in salivary glands before and after RTX treatment

Comparison of immunoglobulin sequences obtained at different time points revealed that clonally-related cells were not only present within biopsies taken at baseline, but that members of these groups of clonally-related cells were also present in biopsies taken after RTX treatment. We did not observe any identical IGHV sequences between different patients.

In total, 42 groups of immunoglobulin sequences from clonally-related cells were obtained both from samples taken at baseline and after RTX. In 35 of these groups, members were found both in samples taken at baseline and at 12-16 weeks after RTX (occurring in 8 out of 11 pSS-RTX patients). In 7 groups, members were found both in samples at baseline and in samples taken at 36-52 weeks after RTX. In one pSS-RTX patient, members of 2 groups of clonally-related immunoglobulin-producing cells were detected at all three time points. A graphical representation of the immunoglobulin sequences from clonally-related cells detected at different time points in 5 pSS-RTX patients is shown in Figure 2. In the two pSS-placebo patients, we observed 5 groups of immunoglobulin sequences from clonally-related cells before and 12-16 weeks after treatment with placebo.

The percentage of sequences belonging to clonally-related cells did not change significantly at 12-16 weeks and 36-52 weeks after treatment with RTX, compared to those at baseline. Both the number of groups as well as clone size at all time points showed the same distribution as observed in pSS patients at baseline (Figure 3).

B cells and plasma cells are present in parotid glands before and after RTX

For some patients, we obtained sections from biopsies collected at all three timepoints. We observed the presence of B cells as well as IgA and IgG-producing cells before and after treatment (Figure 4). Both B cells and plasma cells apparently decreased at 12 weeks after RTX treatment compared to baseline.

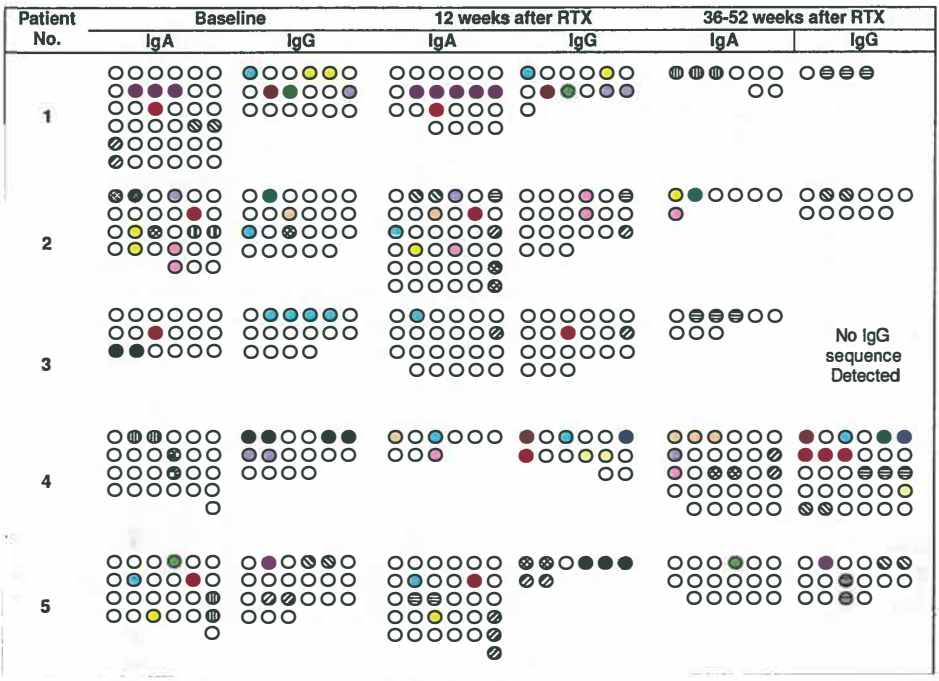


Figure 2: Immunoglobulin sequences from clonally-related cells in parotid salivary glands at baseline and after RTX treatment. Each circle represents the sequence from an Ig-producing cell. This figure shows Ig sequences detected at three different time points in 5 pSS-RTX patients. The black and white shaded circles are clonally-related cells found only at a single time point. Colored circles represent clonally related cells observed at different time points within a particular patient. Note that identical color codes indicate the B-cell clones within each patient and do not imply any B-cell clonality between patients.

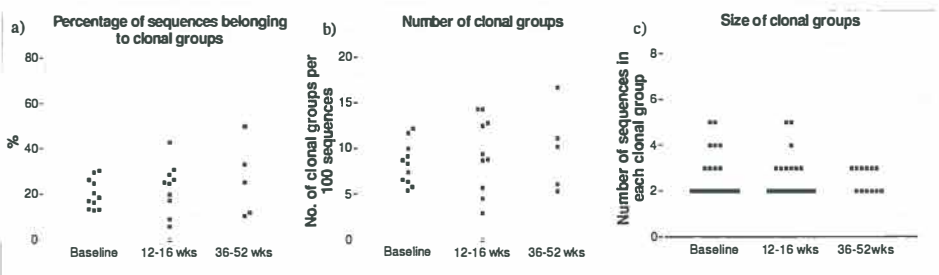


Figure 3: Immunoglobulin sequences from clonally-related cells detected at baseline and after RTX treatment in pSS patients. a) Percentage of immunoglobulin sequences belonging to clonally-related cells in biopsies from pSS patients at baseline compared to those at 12-16 weeks and 36-52 weeks. b) Number of groups of clonally-related cells per biopsy (normalized to 100 sequences/biopsy) in biopsies from pSS patients at baseline compared to those at 12-16 weeks and 36-52 weeks. c) Size of clonal groups in biopsies from pSS patients at baseline compared to those at 12-16 weeks and 36-52 weeks.

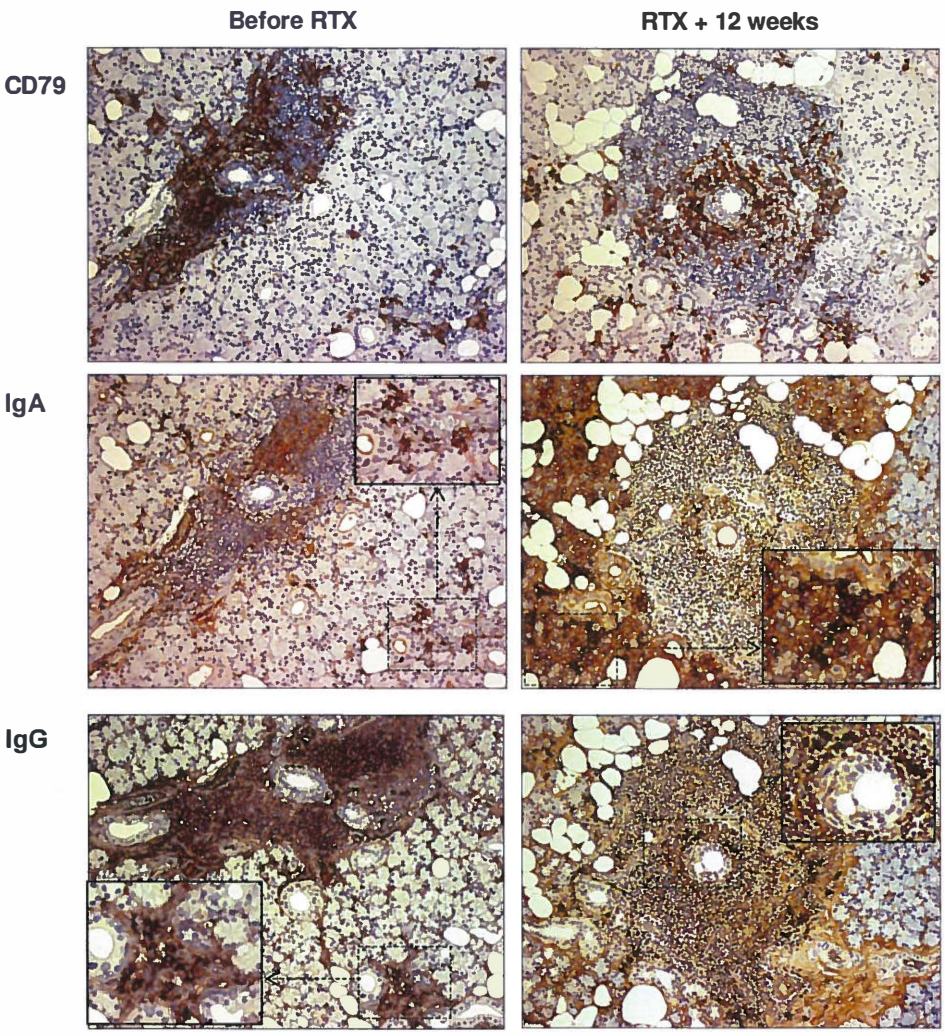


Figure 4: CD79 IgA and IgG-expressing cells in parotid glands from a pSS patient before and after RTX. Sections of a parotid gland were stained with anti-CD79, anti-IgA and anti-IgG antibodies before RTX and at 16 weeks after RTX. B cells as well as IgA and IgG-producing cells were detected at all timepoints (data not shown for 36-52 weeks after RTX).

Increased mutation frequencies in immunoglobulin sequences from clonally-related cells observed after RTX treatment in pSS patients

Virtually all productive immunoglobulin sequences from pSS patients and control patients carried mutations when compared to known germline IGHV genes (1,170/1,172 sequences). Overall, the combined average mutation frequencies of all IGHV sequences (from clonally-

related and unrelated cells) were not significantly different before (11.1 ± 0.49%) and after RTX treatment (10.9 ± 0.38% at 12-16 weeks and 11.0 ± 0.90% at 36-52 weeks after RTX treatment).

However, when only the groups of clonally-related cells were analyzed separately, we observed significantly higher (P = 0.0007, paired t-test) mutation frequencies from cells at 36-52 weeks after RTX treatment than its closest clone (homologous sequence) seen at baseline. An example of a group of sequences from clonally-related cells in pSS-RTX patients at different time points and their differences in mutation patterns is shown in Figures 5a and b.

Evidence for localized immunoglobulin class switching in parotid salivary glands of pSS patients

There were 101 groups of clonally related sequences obtained from biopsies from pSS patients. Thirty-nine percent (39/101) of sequences in these groups included only IgA sequences and another 39% had only IgG sequences. Twenty-three percent (23/101) included members of both isotypes (IgA and IgG), of which 15 groups included immunoglobulin sequences observed at different time points and the remaining eight groups were detected within the same biopsies. These cells probably switched after the somatic hypermutation process occurred as suggested by shared mutations in the IGHV genes.

DISCUSSION

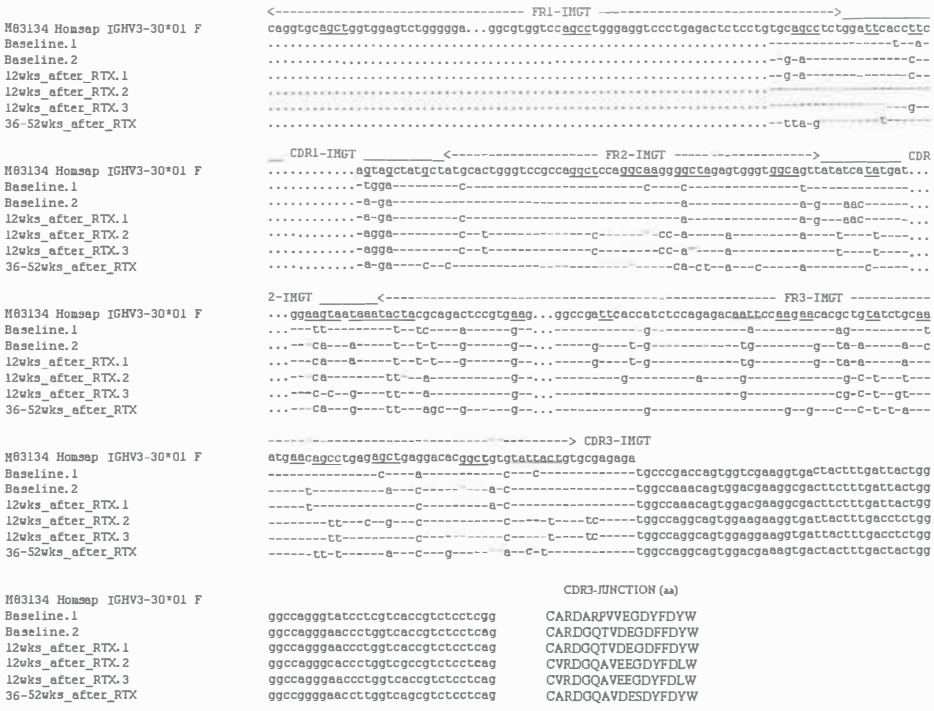
In this study, using IGHV sequence analyses, we provide molecular evidence for the local persistence of certain B-cell populations within the salivary glands of pSS patients after RTX therapy.

Increased clonal expansions of immunoglobulin producing cells are a characteristic feature of parotid glands from pSS patients

Our comparison of the immunoglobulin sequences from biopsies of pSS and control patients indicates that pSS patients can be characterized by the presence of increased clonal expansions within the IgA or IgG-expressing cell populations present in parotid glands. Presence of clonally-related cells has been previously observed in non-inflamed parotid salivary glands in humans (24) and in rats (25). Clonal expansions in salivary glands of pSS patients have also been previously indicated (13-15, 26). Our study adds to these previous reports by including comparisons with non-pSS control patients, thus enabling us to attribute increased clonal expansions as a feature observed within salivary glands of pSS patients. Moreover, we show that the intrinsic property of an increased degree of clonal expansions in pSS patients at baseline was largely unaltered by RTX therapy.

As we analyzed mRNA of entire tissue sections, we did not select for specific immunoglobulin-expressing cell subsets. For this reason it is conceivable that the great majority of the immunoglobulin sequence data obtained reflects the immunoglobulin expression of class-switched plasma blasts or plasma cells, which express approximately 100-1000 fold more immunoglobulin transcripts compared to B-cells (27). On the other hand, this approach has the advantage of providing a greater representation of plasma cell populations, which may include those that actively produce autoantibodies.

It is possible that the biopsy sections from control patients may have much less Ig-expressing cells than pSS patients. We attempted to control for any quantitative bias by picking 36 plasmids for IgA and 36 plasmids for IgG analysis from each biopsy. Even then, due to the amplification biases that may be introduced by PCR, the number of IGHV sequences recovered



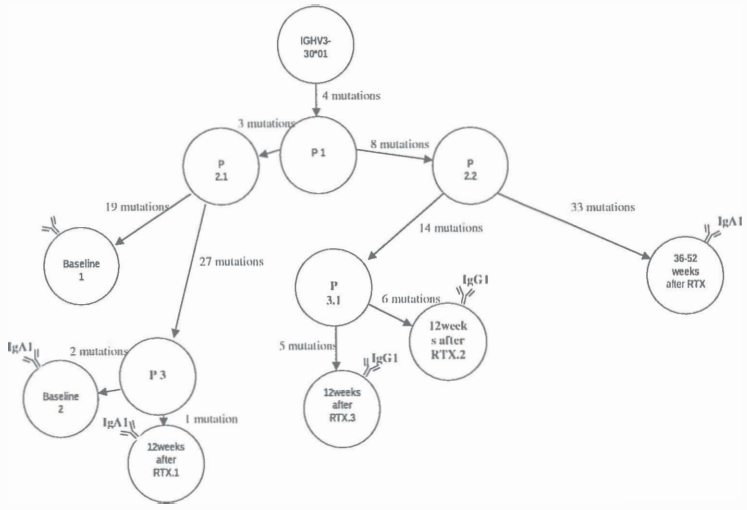


Figure 5: Immunoglobulin sequence alignment of clonally-related cells at baseline and after RTX treatment. An example of six immunoglobulin sequences belonging to persistent clonally-related cells from patient 2 indicated in figure 2 (depicted as pink circles) is shown here. a) Sequences are aligned with the germline gene and their predicted CDR3-junction amino acid (aa) sequences. Dashes indicate identical nucleotides and gaps resulting from IMGT subdivision are marked by dots. b) A genealogical tree is deduced from the mutations observed in the IGHV genes and each cell (sequence) is shown with the observed isotype. P1, P2.1, P2.2 and P3 indicate consecutive hypothetical precursor B-cells that share mutations with the sequences from clonally-related cells.

from these plasmids can only be a rough estimation of the abundance of immunoglobulin-producing cells in the biopsy sections.

We detected immunoglobulin sequences within groups of clonally-related cells expressing different isotypes in pSS patients. Similarly, Dunn-Walters et al (24) had also observed sequences of both IgA and IgM isotypes among members of three groups of clonally-related cells in non-inflamed human parotid salivary glands. In our study, such clonally-related sequences consisted either of combinations of IgG1 and IgA transcripts or of IgA1 and IgA2 transcripts. These findings provide evidence for the existence of localized class-switching within the salivary glands of pSS patients. Since class switching is associated with proliferation, these observations are in line with the presence of clonal expansions, reflecting the hyperactive state of B-cells in affected salivary glands of pSS patients.

Persistence of clonally-related cells before and after treatment with RTX

Our detection of immunoglobulin sequences from clonally-related cells in nearly all (9 out of 11) pSS patients before and after RTX indicates that immunoglobulin-producing cells can persist within the parotid salivary glands even at time points after RTX administration when the peripheral blood is almost completely devoid of B-cells (11). This was also confirmed by immunohistochemical staining of parotid gland tissue sections (Figures 4 and 5). All sequences belonging to groups of clonally-related cells obtained at baseline and after RTX treatment were heavily mutated. Furthermore, at 36-52 weeks after RTX, mutation frequencies of all sequences from clonally-related cells were higher compared to baseline, indicating ongoing proliferation of these immunoglobulin-producing cells. Although we see mutations in both the framework and CDR regions, many of the mutations in the framework region are silent and there is no clear mutation pattern that is consistent with positive selection within CDR regions (data not shown).

All of the above observations give credence to a model of disease relapse after RTX therapy that is seeded by persisting immunoglobulin-producing cells. There are several non-mutually exclusive explanations for the apparent survival of the persistent B-cells/plasma cells after RTX. One possibility is that these could be long-lived plasma cells that do not express CD20 and are therefore not targeted for depletion by RTX (28). Another explanation could be that these are persistent cells derived from memory B-cells that possess a survival advantage to resist RTX depletion (29). Alternatively, the surviving cells may be situated in restricted niches that enable them to evade depletion and proliferate with time. This is a plausible idea, given that the higher mutation patterns observed in immunoglobulin sequence members from clonally-related cells in biopsies taken after RTX. The existence of niches with apparently restricted access to RTX has already been reported in murine systems, where B-cells in certain tissue sites such as the splenic marginal zone, germinal center and peritoneal cavities exhibit significant resistance to anti-CD20 depletion (30, 31). This phenomenon was also observed in the germinal center B-cells of the lymph nodes in non-human primates (32) and in the tonsils(33) and other lymphoid organs in humans (34).

The transient clinical relief from SS symptoms after RTX is probably due to the ablation of B-cell and $CD20^+$ plasma cell numbers (35) resulting in lower levels of certain autoantibodies from short-lived plasma cells and/or reduction in other effector B-cell functions, such as antigen presentation and cytokine production (36, 37). At the same time, the underlying autoimmune mechanisms are probably maintained by long-lived plasma cells as has been indicated in studies in SLE where patients who expressed autoantibodies secreted by long-lived plasma cells, had

a higher chance of experiencing early flares or disease relapse after B-cell depletion therapy than patients who did not express these autoantibodies (36, 38-40). Long-lived plasma cells were also reported in labial salivary glands of pSS patients (41). In this context, our study also provides a robust argument for the development and inclusion of therapeutic strategies that deplete plasmablast and/or plasma cell populations for treating pSS patients (36).

To conclude, we provide evidence for the existence of certain B-cell populations in the parotid salivary glands of pSS patients that may have escaped depletion. The cells that survive after RTX may ultimately contribute to the disease relapse observed in pSS patients who have undergone RTX therapy.

IMMUNOGLOBULIN GENE ANALYSIS REVEALS ALTERED SELECTIVE PRESSURES ON IgG-PRODUCING CELLS IN PAROTID GLANDS OF PRIMARY SJÖGREN'S SYNDROME PATIENTS

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Manuscript submitted

ABSTRACT

Objectives: Patients with primary Sjögren's syndrome (pSS) have increased clonal expansions of B-cells in their parotid salivary glands, that persist after rituximab treatment (RTX). This study aimed at analyzing Ig sequences from pSS patients in order to get insights into possible selective pressures leading to these clonal expansions.

Methods: Five pSS patients received RTX and their parotid salivary gland biopsies were taken at baseline, at 12-16 weeks and at 36-52 weeks after treatment. Parotid biopsies from four non-pSS patients served as controls. Sequence analysis was carried out on IgA and IgG RNA transcripts expressing immunoglobulin heavy-chain variable (IGHV)-3 genes.

Results: Compared to non-pSS controls the following features were significantly increased in pSS patients at baseline: (1) clonal expansions of immunoglobulin-producing cells expressing IGHV-3 (2) expression of IgG1 versus other IgG subclasses (3) conservation of IgG framework regions (4) prevalence of acquired N-glycosylation sites in the variable heavy-chain regions of IgG sequences. All these typical characteristics of immunoglobulins from pSS patients were unaffected by RTX.

Conclusions: We observed fundamental differences in selective pressures and frequency of acquired N-glycosylation sites in IgG sequences derived from parotid glands of pSS patients versus non-pSS samples. Furthermore, we showed that B-cell depletion using RTX did not affect these characteristics in RTX-treated pSS patients. As the cells that persist after RTX may contribute to disease relapse after RTX, our data may have significant implications for the rationale of using therapies targeting Ig-producing cells in conjunction with B-cell depletion treatment in pSS patients.

Keywords: Sjögren's syndrome, autoimmune, B-cell depletion, immunoglobulin

Sjögren's syndrome (SS) is an autoimmune disorder that primarily affects the salivary and lacrimal glands. The main clinical features are progressive dryness of mouth and eyes. If these symptoms occur without other underlying autoimmune conditions, the disease is called primary SS (pSS) (1).

Lymphocytic infiltrates composed of T-cells, B-cells and plasma cells are present in the salivary glands of pSS patients (2) and are organized as ectopic lymphoid tissue, sometimes containing germinal center (GC)-like structures (3). Disturbances in relative proportions of peripheral B-cell subsets and high titers of circulating autoantibodies such as anti-Ro (SS-A) and anti-La (SS-B) in the blood are key features of pSS (4). There is also an increased risk for the development of B-cell malignancies in pSS patients (3). Furthermore, therapeutic B-cell depletion strategies targeting CD20 provide clinical relief from pSS symptoms (5,6). However, symptoms usually return 6-9 months after treatment and disease relapse seemingly coincides with the reappearance of peripheral B-cell subpopulations in the blood (6,7). All the above observations strongly suggest that B-cells and/or plasma cells play a significant role in the disease mechanisms underlying pSS, although their exact role in pSS pathogenesis is still undefined.

We previously reported the persistence of certain clonal populations of immunoglobulin (Ig)-producing cells despite B-cell depletion with rituximab treatment (RTX) (8). One obvious reason for this could be the widely believed lack of CD20 expression on plasma cells. However, there are reports of CD20+ plasma cells being present in tonsils even after RTX (9). We also showed that B-cells persisting after RTX were more mutated in their immunoglobulin heavy-chain variable (IGHV) genes than their clonally-related counterparts present before RTX (8). This is an indication that Ig-producing cells surviving after RTX continue to proliferate. However, the factors promoting selection, survival and expansion of these Ig-producing cells are still unclear.

This study aimed at analyzing Ig sequences to obtain insights into possible selective pressures leading to increased clonal expansions of Ig-producing cells in pSS patients. We observed that Ig sequences from pSS patients differed from those in non-pSS controls with respect to isotype subclass usage, conservation of framework regions and presence of acquired N-glycosylation sites. Furthermore, we showed that B-cell depletion using RTX does not alter these characteristics in treated pSS patients. Our data may have significant implications for the rationale of using therapies targeting Ig-producing cells in conjunction with B-cell depletion treatment in pSS patients.

MATERIAL AND METHODS

Patients

Five pSS-patients (all females; mean age 49.5 years; range 36-65 years) with a disease duration of less than 5 years, were enrolled in this study after informed consent. These patients fulfilled the 2002 American-European criteria (10) and the recently published American College of Rheumatology (ACR) criteria for pSS (11). Inclusion criteria of the pSS-patients used in this study were as mentioned before (6): stimulated whole saliva secretion flow >0.15 ml/min, presence of autoantibodies (IgM-rheumatoid factor ≥10 kIU/liter in combination with anti-SS-A and/or anti-SS-B autoantibodies), and a salivary parotid gland biopsy (obtained ≤12 months before inclusion) showing characteristic features for SS (12). The 5 pSS patients were treated with RTX as described before (6). All patients showed significant depletion of B-cells in the peripheral blood at 12 weeks after RTX. In all pSS patients, incisional biopsies of the parotid gland were obtained from the same gland before RTX, as well as at 12-16 weeks and 36-52 weeks after RTX. The biopsies were frozen in liquid nitrogen immediately after surgery.

In addition to the 5 pSS patients, 2 patients with sicca complaints not fulfilling the American-European and ACR criteria for pSS and 2 patients with malignancies (squamous cell carcinoma of the oral cavity) without involvement of the parotid salivary glands were included as non-pSS controls. From these 4 non-pSS controls, we obtained single parotid biopsies with laboratory-confirmed normal histology. The study protocol was approved by the institutional review board of the University Medical Center Groningen.

Cloning and sequencing of immunoglobulin transcripts

From every biopsy belonging to pSS patients and non-pSS controls, total RNA was extracted. These RNA samples were converted to cDNA and amplified using primers specific for the IGHV3 gene (13) in combination with constant region primers specific for the CH1 domains of the Cα (14) or Cγ (8) constant regions.

PCR and cloning was performed as reported previously (8). A total of 72 (36 IgA + 36 IgG) plasmid constructs were picked separately for each biopsy (after checking for PCR product insertion) and submitted for sequencing.

Analysis of immunoglobulin transcript sequences

The immunoglobulin variable regions and isotype subclasses were identified using the IMGT databases (15). Identical/similar sequences (different by ≤ 2 mutations to account for Taq error-rate) derived from the same PCR were considered redundant and counted as one. Only productive sequences (encoding functional proteins) were included in this study. Ig-producing cells were considered as clonally-related (hereafter referred to as clones) based on their similarity of nucleotide sequence at the complementarity determining region (CDR) 3, their shared IGHV-D-J gene usage, identical/similar N1 and N2 additions and their pattern of shared mutations within Ig variable regions (16).

The degree of clonal expansions of Ig-producing cells was calculated as the percentage of sequences belonging to a clone among all sequences. For analyzing the diversity of IGHV3 gene usage, each clone was represented by a single sequence member and this set was referred to as unique sequences. This approach eliminated skewing of data due to sequences from expanded clonal populations. For isotype subclass usage, we analyzed all sequences, including all members of clones and not just unique sequences as we observed several clones with members of different isotypes. Excluding clonally-related sequences would have biased for or against a certain isotype.

Analysis of selection pressures

Selection pressure analysis was carried out with an online program known as the Focused test (17) which predicts the type and extent of selection pressures that shape the Ig repertoire on the basis of mutation patterns. The Focused test compares observed frequencies of replacement (non-synonymous) or R mutations and silent (synonymous) or S mutations relative to their expected frequencies calculated under the null hypothesis of no selection. The statistical significance for these tests are determined by a binomial test ($p = 0.05$). The Focused test analyzes regions separately and does not allow statistical cross-talk between CDRs and framework regions (FRs) (17). For prediction of selection pressures based on somatic hypermutation (SHM) patterns, we included all sequences except those with insertions and/or deletions.

Prediction of potential N-glycosylation sites

The prediction of potential N-glycosylation sites was carried out on amino acid (aa) sequences of the entire variable heavy-chain (IGHV-D-J). N-Glycosylation sites were predicted using the NetNGlyc 1.0 online tool (http://www.cbs.dtu.dk/services/NetNGlyc/). N-glycosylation is known to occur on asparagines (N) which occur within an N-X-serine (S) /threonine (T) motif (where X is any aa except proline as it precludes N-glycosylation due to steric hindrance). We also excluded the motif N-X-S if tryptophan, aspartic acid, glutamic acid and leucine were at position X, as these are poor oligosaccharide acceptor motifs (18). The criteria set for accepting prediction of N-glycosylation sites were Potential > 0.5 and Jury agreement ≥ 5/9 (19). For N-glycosylation site prediction, we analyzed all sequences, including clonal sequences as we observed several clones with members of different isotypes.

Statistical analysis

Data from ifferent groups were statistically compared using GraphPad Prism software (version 3.0; http://www.graphpad.com/). Unpaired t-test was used for comparing pSS patient and non-pSS control data. Data from baseline and after treatment in pSS patients were analyzed using paired t-test. Dispersion of values is numerically reported as mean ± standard deviation.

RESULTS

Increased clonal expansions of IGHV3-expressing clones

We analyzed a total of 543 productive IGHV3 sequences from three timepoints in 5 pSS patients and 93 IGHV3 sequences from 4 non-pSS controls (Supplementary data, Table A). The degree of clonal expansions (expressed as percentage of sequences belonging to a clone among all sequences) within IGHV3-expressing cell populations was significantly higher (p=0.001; Figure 1) in pSS patients at baseline than in non-pSS controls. RTX did not affect this pattern of increased clonal expansions in pSS patients.

In the 5 pSS patients, we observed a total of 23 clones whose members were present both before and after treatment. Of these 23 clones, 2 clones had member sequences with the IgA isotype, while 7 clones had members expressing both IgA and IgG isotypes. In the 4 non-pSS controls, we observed only a single clone with 2 members belonging to the IgA isotype. When the diversity of IGHV3 gene usage in the parotid glands of pSS patients was compared to non-pSS controls, we did not observe a strong bias for usage of a particular IGHV3 gene in pSS patients (Supplementary data, Figure S1a). The diversity of IGHV3 gene usage observed in pSS patients at baseline was unaffected by RTX (Supplementary data, Figure S1b). There was also no difference in IGHV3 gene usage between IgG versus IgA-encoding sequences.

IgG isotype subclass usage differ in pSS patients and non-pSS controls

We analyzed for differences in subclass usage among IgG sequences between pSS patients and non-pSS controls. The predominance of certain subclasses may indicate a selection of particular effector functions by the persisting Ig-producing cells and/or their antibodies.

The distribution pattern of IgG subclasses was significantly different between pSS patients and non-pSS patients (Figure 2). In pSS patients at baseline, the relative IgG1 subclass usage (88.6±15.9%) was significantly higher (p=0.0015) than in non-pSS controls (31.0±14.4%). In

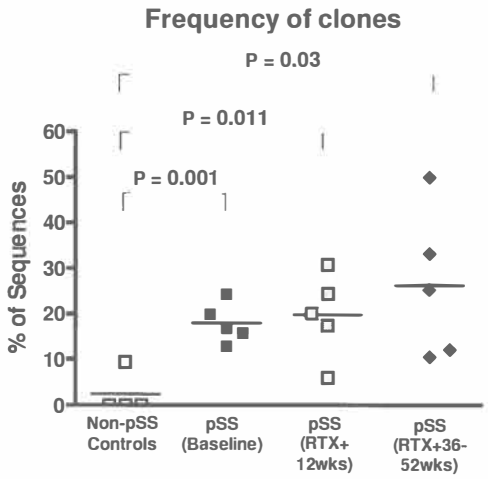

Figure 1: Clonal expansions are increased in pSS patients. The frequency of immunoglobulin sequences belonging to clonally-related cells among all sequences in parotid gland biopsies of pSS patients (at baseline, 12weeks after RTX and 36-52 weeks after RTX) compared to the frequency in parotid gland biopsies of non pSS controls.

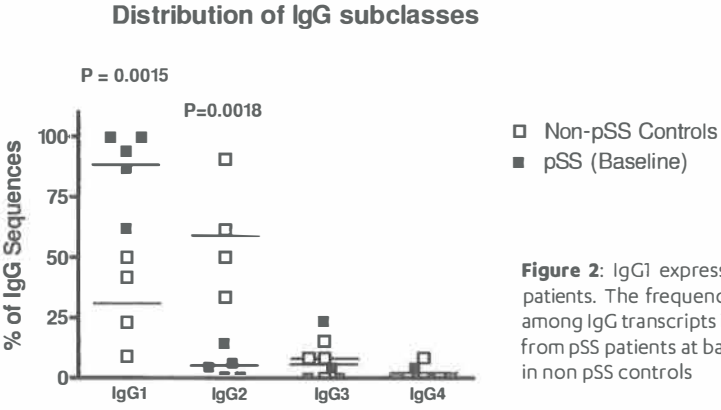

Figure 2: IgG1 expression is increased in pSS patients. The frequency of IgG1 isotype usage among IgG transcripts in parotid gland biopsies from pSS patients at baseline compared to that in non pSS controls

non-pSS controls, on the other hand, IgG2 was the dominant IgG subclass (p=0.0018). The higher expression of IgG1 isotype subclass in pSS patients at baseline was not significantly altered by RTX (95.2 ±10.6% at 12 weeks after RTX and 76.9±43.5% at 36-52 weeks after RTX). The levels of IgG3 and IgG4 expression were low in both pSS patients and non-pSS controls and did not change after RTX (data not shown).

Prediction of antigen selection pressure

Affinity maturation is a process by which B-cells accumulate somatic mutations within their immunoglobulin genes after which, they are selected based on their affinity to antigens. The distribution of replacement versus silent (R/S) mutations, is used by the Focused test (17) to determine the role of antigen selection in shaping the Ig repertoire. In the Focused test, the presence of significantly high R/S ratios in a sequence is indicated as 'positive antigen selection'. On the other hand, significantly low R/S ratios would indicate a sequence that is selected against major changes at the protein level (also referred to as 'negative selection' by the Focused test) (17).

Despite the fact that all immunoglobulin sequences obtained from pSS patients were highly mutated, only 9.6±2.8% of the IgG sequences and 6.1±3.3% of the IgA sequences from pSS patients at baseline were predicted by the Focused test to exhibit significant positive antigen selection (higher R/S) in their CDRs. This was similar to the patterns observed in non-pSS controls (6.8±3.8% for IgG and 8.9±6.5% for IgA) and in pSS patients at 12 weeks after RTX (4.8±4.9% for IgG and 3.9±3.3% for IgA). However, at 36-52 weeks after RTX, the percentages of IgG and IgA sequences showing positive selection in their CDRs decreased compared to baseline values (3.2±4.4% for IgG and 0.8±1.9% for IgA), but this decrease was statistically significant only for IgG ($p=0.035$; Figure 3a). The frequency of IgG and IgA sequences from pSS patients at baseline showing 'negative antigen selection' in their CDRs (lower R/S) was also low and similar to non-pSS controls (data not shown) and no significant change was observed in pSS patients after RTX (data not shown). This suggests that despite having mutated CDRs, the selection of Ig-producing cells is not significantly dependent on their CDRs in both pSS patients and non-pSS controls.

None of the IgG and IgA sequences showed positive selection in their FRs, which was not surprising, since the FRs essentially encode for the structural backbone of immunoglobulins. However, the frequency of IgG sequences showing negative selection (less R/S, implying conservation of structure) in their FRs was significantly higher ($p=0.013$) in pSS patients at baseline than in non-pSS controls (Figure 3b). There was no change to this pattern in pSS patients after RTX when compared to baseline.

Our results indicate that the selection pressures acting on the FRs of IgG sequences in pSS patients are significantly different from those in non-pSS controls. The frequency of IgA sequences showing negative selection in their FRs did not differ between pSS patients at baseline and non-pSS controls or after RTX (data not shown).

Incidence of N-glycosylation sites in IGHV3 regions

All analysed sequences exhibited somatic mutations. However, a surprisingly low percentage of sequences showed evidence for positive antigen selection within CDRs which are the conventional antigen-binding regions. This suggests the possible involvement of other factors that may promote selection of certain Ig-producing cells in pSS patients. We evaluated

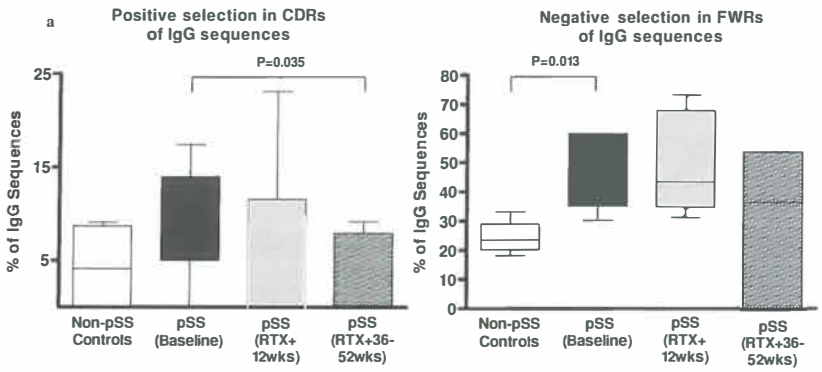

Figure 3: Selection pressures on IGHV regions of IgG sequences from pSS patients. The panel indicates (a) levels of antigen selection in CDRs of IgG sequences from pSS and non-pSS controls (b) negative selection (maintenance of structural integrity) in FWRs of IgG sequences in pSS patients compared to non-pSS controls.

if N-glycosylation could be one of these factors because the acquisition of N-glycosylation motifs by somatic hypermutation was suggested to confer a selective advantage to certain B cells in a model system simulating GC reactions (20).

The prevalence of IgG sequences with N-glycosylation sites was significantly higher (p=0.01; Figure 4) in pSS patients at baseline (24.5±11.9) compared to non-pSS controls (4.0±4.6). It was not feasible to analyse any IgG subclass-related differences in N-glycosylation sites since there were very few IgG2, IgG3 and IgG4 sequences in pSS patients. No such bias was observed within the IgA sequences of pSS patients at baseline (14.3±4.5) compared to non-pSS controls (18.8±20.9) (data not shown). Also, when compared to baseline, the distribution of N-glycosylation sites did not change significantly in IgG or IgA sequences derived from pSS patients after RTX.

Of the 23 clones found both before and after RTX in pSS patients, 13 clones had all or some members with N-glycosylation sites. Among these 13 clones, 8 belonged to the IgG isotype and 5 clones expressed the IgA isotype. The majority of N-glycosylation sites (93/95) detected in this study was a result of somatic mutations and hence acquired motifs. Of the 2 naturally-occurring (germline-encoded) N-glycosylation sites, one was detected in a non-pSS control and the other, in a sequence from a pSS patient at 36-52 weeks after RTX (Table 1).

To further delineate the differences in acquired N-glycosylation patterns between pSS patients and non-pSS controls, we analyzed the aa motifs encoding the N-glycosylation sites in all sequences obtained. Among the 44 Ig sequences from pSS patients at baseline carrying an acquired N-glycosylation site, 26/44 sequences (~60%) had sites in the FR3 region. Seven of these FR3 N-glycosylated sequences belonged to 5 different clones detected both before and after RTX. The percentage of FR3-associated N-glycosylation sites were 55% (11/20) among IgA and 62.5% (15/24) among IgG sequences. The bias for N-glycosylation sites in the FR3 was also seen in pSS patients after RTX.

Of these 26 sites noted in FR3s in pSS patients at baseline, 19 (76%) sites were created by mutations at residue 84 and the remaining mutations were at residues 77 and 93 (3 sequences each). In IGHV3 genes, the mutations forming N-glycosylation sites at residue 77, 84 and 93 created changes from (germline-encoded) threonine, lysine and serine respectively, to asparagine, all of which are hydrophilic aa. This indicates an emphasis on the conservation of aa properties at residues 77, 84 and 93 of the FR3.

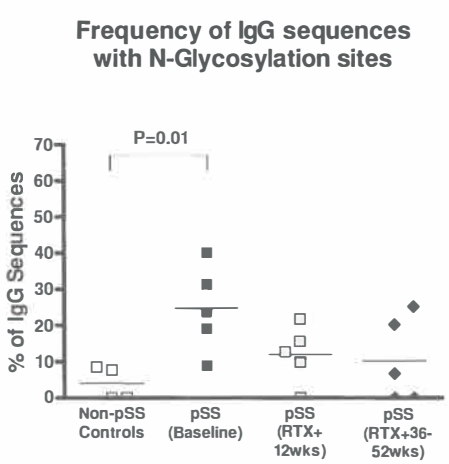

Figure 4: Incidence of acquired N-glycosylation sites is increased in pSS patients. The frequency of acquired N-glycosylation sites among IgG transcripts from parotid gland biopsies of pSS patients (at baseline, 12weeks after RTX and 36-52 weeks after RTX) compared to that in non pSS control biopsies.

Table 1: The number of Ig sequences analyzed in this study

Sample group	Patient	FR1	CDR1	FR2	CDR2	FR3	CDR3
Non-pSS comtrols	NC-1		NFS,				
	NC-2				NIT	NAT **(n)**	
	NC-3		NSS, NSS			NNT	NVT, NRT
	NC-4		NFS			NKS	
pSS (Baseline)	pSS-1		NIS, NVS			NDT,NGS,NNT,NIS,NHT, NNT, NIS, NNT, NKT, NGS, NGT	NIS, NGT
	pSS-2		NSS			NNT, NNT	NVT
	pSS-3		NSS, NYS			NNT, NNT, NNT, NNS, NIS, NLT,NNT	
	pSS-4		NFT, NFS		NGT, NYT,	NYS, NYS, NTT	NNT
	pSS-5		NYS, NFS	NIS	NTT, NTS	NDT, NDT,NNT	
pSS (RTX+12wks)	pSS-1		NFS,NIS			NNT, NST, NNT, NNT, NNT	NGT, NGT
	pSS-2				NGT, NGT	NNT	
	pSS-3					NNT, NNT	
	pSS-4		NVS, NFS			NTT, NNT, NFS	NGT
	pSS-5		NVS, NFT			NDT, NDT, NNS, NNT	NAT, NYS
pSS (RTX+36-52wks)	pSS-1		NRT			NAS **(n)**	
	pSS-2						
	pSS-3				NRT		
	pSS-4		NIS, NVT			NNT, NTT, NNT, NNS, NYT	NGT
	pSS-5	NTS	NFS			NVT, NDT	

The number of IgG and IgA sequences from non-pSS controls and from pSS patients at baseline, at 12 weeks and at 36-52 weeks after RTX. A total of 543 productive IGHV3 immunoglobulin sequences were analyzed from 5 pSS patients.

DISCUSSION

The results of this study clearly indicate that Ig sequences in pSS patients exhibit certain core characteristic features, such as increased clonal expansions, increased IgG1 usage, a higher emphasis on IgG framework region structural integrity and an increased incidence of N-glycosylation motifs in their IgG sequences, all of which distinguish them from non-pSS controls. These features were not affected by B-cell depletion using RTX.

As we analyzed mRNA sequences and did not select for specific B-cell subsets, it is conceivable that much of our Ig sequence data obtained reflected the expression of class-

switched plasma blasts or plasma cells, which are known to express approximately 100-1000 fold more immunoglobulin transcripts compared to B-cells. On the other hand, this approach has the advantage of providing a greater representation of Ig-producing cells that are active producers of (auto)antibodies such as anti-SSA/Ro and anti-SSB/La antibodies which may be produced by plasma cells located in glandular tissues of pSS patients (21).

IGHV3-expressing clonal expansions are significantly higher in pSS patients

We previously reported the presence of clonal populations within parotid glands of pSS patients which persisted after B-cell depletion with RTX (8). In that study, we compared between IgA sequences from pSS patients and non-pSS controls, irrespective of IGHV gene family usage. We took our analysis further in this study by analyzing both IgA and IgG sequences expressing IGHV3 genes in pSS patients and non-pSS controls. We confirmed that increased clonal expansions of cells expressing IGHV3 genes were a feature more characteristic of pSS patients as opposed to non-pSS controls. Ongoing studies by our group will verify whether clonal expansions in pSS patients are restricted to the IGHV3 family or are also seen in other IGHV gene families.

Higher IgG1 expression in pSS patients

To our knowledge, this study is the first to show a significantly higher expression of IgG1 transcripts over other IgG suclasses in the parotid glands of pSS patients as compared to those in non-pSS controls. These findings are in line with a previous report that levels of IgG1 immunoglobulins are increased in the saliva of pSS patients compared to healthy controls (22).

In various autoimmune disorders, autoreactive antibodies are predominantly of the IgG1 isotype. Anti-SS-A/Ro autoantibodies from pSS patients (23) and anti-SS-B/La autoantibodies from pSS and systemic lupus erythrematosus (SLE) patients (24, 25) as well as the autoantibody repertoire in diseases such as bullous pemphigoid (26), myasthenia gravis (27), rheumatoid arthritis (28) and SLE (29) show marked IgG subclass restriction in favor of IgG1. Moreover, autoantibodies to neutrophil cytoplasmic antigens in SLE (30) and ulcerative colitis patients (31) mostly belong to the IgG1 isotype.

IgG1 is thought to be more effective at phagocytosis and complement-fixation than IgG2 (32). Hence, the relative predominance of IgG1-expressing cells which possibly bind (auto) antigens within the diseased salivary glands of pSS patients, may attract a powerful complement-mediated detrimental immune response locally, leading to the pathology observed in the salivary glands of pSS patients.

Emphasis on maintaining immunoglobulin structural integrity rather than on antigen selection in pSS patients

Affinity maturation is a process within GCs where mutations are introduced into the rearranged Ig genes in B-cells, followed by selection of B-cells producing immunoglobulins with higher antigen-affinity. The distribution of R and S mutations that remain after affinity maturation is used by the recently developed Focused test to investigate the role of antigen selection in optimizing the Ig repertoire (17). The suitability of the Focused test to evaluate positive antigen selection was effectively demonstrated by a study on Ig sequences obtained from antigen specific B-cells during primary and memory immune response in immunized mice (33).

An important observation in our study was that despite being highly mutated, a relatively low number of sequences showed signs of significant positive selection in IgA and IgG sequences from either pSS patients at baseline or non-pSS controls. Similar findings were also reported by a

previous study which analyzed Ig sequences from salivary glands, lymph nodes, peripheral blood and bone marrow of pSS patients and in tissues from patients with other autoimmune disorders (RA and multiple sclerosis) (34). Moreover, in our study, the frequency of IgG sequences showing positive antigen selection decreased even further at 36-52 weeks after RTX (Figure 4a). Since we noted significant positive selection in only a few sequences, we do not have strong evidence for the role of antigen selection on the Ig repertoire in pSS patients and non-pSS controls. These observations are in contrast to the conclusions of other studies which assume antigen-driven clonal B-cell expansions in pSS based on higher R/S ratios of somatic mutations (35, 36). However, these studies did not differentiate between mutations introduced during affinity maturation and mutations that occur due to the bias for mutational hotspots as well as the differences in selection pressures between CDRs and FRs. All of these factors are considered within the Focused test (17).

In addition to this, we also observed an increased emphasis on the maintenance of structural integrity (negative selection) in the FRs of IgG sequences from pSS patients compared to non-pSS controls. The above observations raise the possibility that Ig-producing cells in autoimmune repertoires may be selected by unconventional processes that may not involve classical GCs for generation of somatically hypermutated B-cells (37, 38).

Significantly higher incidence of acquired N-glycosylation sites in IgG sequences from pSS patients

Given the minimal evidence for positive antigen selection pressure on the Ig sequences from the parotid glands of pSS patients, we speculate that these sequences may confer a selective advantage on B-cells based on certain properties of the immunoglobulins produced rather than on the specificity of antibody-antigen interactions. In this regard, studies on acquired N-glycosylation sites within the Ig variable regions in B-cell malignancies provided interesting clues as to a potential role for N-glycosylation in conferring a selective advantage to B-cells (20, 39-41).

During the modulation of the Ig repertoire, the SHM process may introduce mutations in the variable regions of both heavy and light chains that either create new (acquired) N-glycosylation sites or destroy such sites present in a germline variable gene. Studies on immunoglobulin variable (V) regions revealed higher frequencies of acquired N-glycosylation sites in follicular lymphomas (FL) compared to other B-cell malignancies and normal somatically mutated memory B-cells (39, 42). The relative lack of N-glycosylation motifs in the V-regions of normal memory B-cells, normal IgM and IgA plasma cells and non-FL B-cell lymphomas suggests that the frequency of N-glycosylation sites is generally quite low in post-GC Ig-producing cells (39). In contrast, we observed that a high proportion (~25%) of the IgG sequences from parotid glands of pSS patients exhibited acquired N-glycosylation sites in their IGHV regions. This suggests that some IgG-producing cells in the parotid glands of pSS patients may have been selected on the basis of their glycosylation patterns.

Sixty percent of the acquired N-glycosylation motifs in pSS patients both before and after RTX, were created by replacement mutations at residues 77, 84 and 93 within the FR3s of Ig sequences. However, at all three sites, the acquired asparagine residue and the replaced germline-encoded residues, were all hydrophilic, indicating the conservation of core aa properties. This is in line with our earlier discussion on the increased emphasis on the maintenance of FR structural integrity.

Interestingly, immunoglobulin FRs are associated with B-cell activation through their binding by superantigens (sAg). Superantigens such as the Staphylococcal protein A (SpA), the endogenous human gut-associated sialoprotein pFv and the HIV-1 envelope protein

gp120 interact with the evolutionarily conserved IGHV framework regions and stimulate B-cell differentiation and Ig secretion (43). SpA interacts specifically with IGHV3 sequences between residues 75 and 84 of the FR3 region (44). The gp120 and pFv proteins also share a similar affinity to the same or nearby FR3 sites, as was shown by competitive binding experiments with SpA (43, 45). Binding of superantigens to these FR3 sites results in B-cell activation independent of conventional antigen binding sites in the CDRs. Hence, the emphasis on maintaining the overall structure of FRs outside of the classical antigen-binding sites may also be a sign of B-cell selection. Remarkably, these sAg-interacting FR3 sites are at the same location where we observed nearly 60% of all N-glycosylation motifs (residues 77, 84 and 93) occurring within Ig sequences from pSS patients. This suggests that these FR3 associated N-glycosylation sites in Ig sequences from pSS patients might well be involved in B-cell activation by molecules that bind to these sugar motifs, such as lectins (46).

Lectins are not only expressed on the surface of microbial antigens, but also on components of the microenvironment, such as T and NK cells (47, 48) as well as macrophages and dendritic cells (49). Interestingly, lectins are also present on acinar and luminal cells of the secretory ducts within salivary glands (50). Interactions with lectins may drive B-cell proliferation and ongoing SHM within parotid glands of pSS patients in a non-classical way, independent of antigen-binding specificity. Such interactions may also occur outside GCs, as suggested by William et al. who showed evidence for proliferation, SHM and (auto)antibody formation outside classical GCs in murine spleen (38). Furthermore, the significant prevalence of IgG sequences with N-glycosylation sites indicates that isotype may play an as yet undefined, but vital role in the selection and survival of these Ig-producing cells in pSS patients.

To conclude, there are clear differences in the characteristics of IGHV3-expressing IgG-producing cells from parotid glands of pSS patients versus non-pSS controls. We observed increased IgG1 subclass expression and an increased emphasis on maintenance of immunoglobulin FR structural integrity in pSS patients. To our knowledge, this is the first study to suggest a role for N-glycosylation in the selection of Ig-producing cells in pSS patients. We postulate that a proportion of B-cells in pSS patients may be selected on the basis of acquired N-glycosylation motifs in their Ig variable regions. B-cell depletion with RTX failed to reset these core characteristics of Ig-producing cell populations in treated pSS patients. Hence, Ig-producing cells that persist after RTX (8) may contribute to the disease relapse observed after RTX (6).

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SUPPLEMENTARY DATA

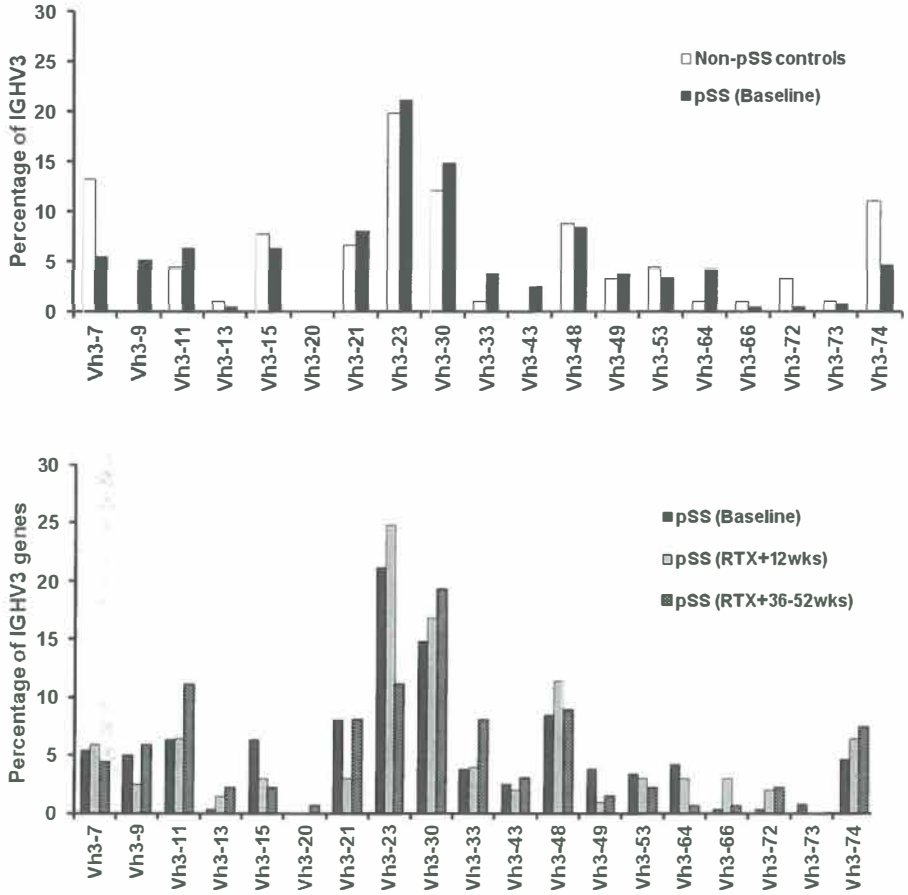

Figure S1: Distribution of IGHV3 gene usage. Comparison of IGHV3 gene distribution in (a) pSS patients and non-pSS controls (b) pSS patients before RTX, at 12 weeks after RTX (RTX+12wks) and at 36-52 weeks after RTX (RTX+36-52wks).

Table 1: The number of Ig sequences analyzed in this study

Sample group	No. of IgA sequences	No. of IgG sequences	Total no. of sequences
Non-pSS controls (n=4)	45	48	93
pSS (Baseline) (n=5)	132	97	229
pSS (RTX+12wks) (n=5)	112	72	184
pSS (RTX+36-52wks) (n=5)	72	58	130

The number of IgG and IgA sequences from non-pSS controls and from pSS patients at baseline, at 12 weeks and at 36-52 weeks after RTX. A total of 543 productive IGHV3 immunoglobulin sequences were analyzed from 5 pSS patients.

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ACQUIRED N-GLYCOSYLATION OF IMMUNOGLOBULINS IN SYSTEMIC AUTOIMMUNE DISEASES MAY MIMIC B CELL-SUPERANTIGEN INTERACTIONS

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Manuscript in preparation

ABSTRACT

Objectives: We previously reported significantly increased prevalence of N-glycosylation sites (Nglyc) in IgG sequences from parotid salivary glands of patients with primary Sjogren's syndrome (pSS), an autoimmune disease. This persisted even after B-cell depletion treatment with Rituximab (RTX). We undertook this study to evaluate if aquired N-glycosylation sites created by somatic hypermutation (SHM) occurred in autoimmune diseases other than pSS.

Methods: We conducted a meta-analysis of acquired N-glycosylation sites on IGHV sequence data from our previous studies in pSS and other published datasets from multiple sclerosis (MS), RA, SLE, Chagas's Disease (ChD), Ankylosing spondylitis (AS) and Wegener's granulomatosus (WG). As controls, we collected published datasets of IGHV sequences derived from various normal and non-autoimmune tissues as well as from vaccination or infection studies on antigen-specific B cells. The amino acid sequences of all Ig sequences were run through the online N-glycosylation site prediction program NetNglyc 1.0.

Results: Overall, the prevalence of acquired N-glycosylation sites was significantly higher in the autoimmune repertoire when compared to those from normal tissues and antigen-specific datasets and this difference was more evident in IgG sequences from autoimmune diseases. Moreover, in autoimmune diseases, the prevalence of acquired N-glycosylation sites in the framework regions (FR) of IGHV sequences was significantly greater than that in normal controls and antigen-specific datasets. In contrast, the majority of acquired N-glycosylation sites in antigen-selected sequences occurred in complimentarity-determining regions (CDR). We also noted that the majority of acquired N-glycosylation sites within FRs coincided with those targeted by superantigens that interact and activate B cells.

Conclusions: Our study clearly indicates a significant selection of B cells with ac-Nglycs in autoimmune diseases compared to normal controls and antigen-specific B-cells. This selection is even more evident within IgG-producing cell populations. The explicit tendency for Ig sequences from autoimmune diseases to acquire N-glycosylation sites within the FRs is in contrast to antigen-selected sequences, where the majority of acquired N-glycosylation sites occur in CDRs. This strongly suggests that acquired N-glycosylation of Fab regions of B cells in autoimmune diseases may be a form of selection more homologous to B cell-superantigen interactions which also occur on FRs. Our study has implications for the future development of evolving therapies for systemic autoimmune diseases.

INTRODUCTION

Autoimmune diseases are conditions where the body mounts immune responses against its own organs or tissues. As most autoimmune diseases are accompanied by an excessive production of antibodies that apparently bind to self-antigens, immunoglobulin (Ig)-producing cells (B-cells and plasma cells) likely play a significant role in perpetuating autoimmunity. Moreover, B cell depletion strategies in certain systemic autoimmune diseases have resulted in temporary clinical relief, with a recurrence of diseases symptoms occurring parallel to the reappearance of circulating peripheral B cells (1, 2). However, the precise mechanisms underlying selection and proliferation of B cells in autoimmune diseases are unclear.

We previously reported low frequencies of Ig-producing cells with evidence for antigen-driven selection in primary Sjogren's syndrome (pSS) [Hamza et al, submitted]. In our search for alternative selection mechanisms, we observed significantly increased prevalence of acquired N-glycosylation sites (Nglyc) in immunoglobulin heavy-chain variable (IGHV) regions of IgG in pSS patients compared to non-pSS controls. This persisted even after B-cell depletion treatment with Rituximab (RTX) [Hamza et al, submitted]. During the modulation of the Ig repertoire, the somatic hypermutation (SHM) machinery may introduce mutations in heavy and light-chain variable regions, that either create new (acquired) Nglycs or destroy naturally-occurring Nglycs in germline variable genes. However, the observation of a significant frequency of Ig-producing cells with acquired Nglycs in pSS was in contrast to the fact that normal memory B-cells and normal plasma cells generally exhibit a relative lack of Nglycs in their variable regions (3).

We undertook this study to evaluate if SHM-related aquired N-glycosylation sites (ac-Nglycs) occurred in autoimmune diseases other than pSS. Such an observation may be indicative of novel B cell selection mechanisms that shape the systemic autoimmune Ig repertoire and may point to mechanisms that underlie the pathology of systemic autoimmune diseases.

METHODS

We analyzed IGHV sequence data from our previous studies in pSS and other published datasets from multiple sclerosis (MS), rheumatoid arthritis (RA), systemic lupus erthrematosus (SLE), Chagas's Disease (ChD), ankylosing spondylitis (AS) and wegener's granulomatosus (WG). As controls, we collected published datasets of IGHV sequences derived from various normal and non-autoimmune tissues as well as antigen-specific sequences from vaccination or infection studies. (Tables 1a and 1b). All IGHV sequences were compared with the international ImMunoGeneTics (IMGT) databases of Ig germline sequences by only using the high-throughput tool, High V-Quest (4), since the IGHV gene identification was(5) different when the low-throughput IMGT V-quest was used, particularly in sequences with ambiguous nucleotide reads. All sequences that were unproductive, non-Ig and identical or redundant sequences were eliminated from our analyses. Clonal sequences with ≤ 2 unshared mutations were also excluded if they expressed identical isotypes. As we wanted to compare the prevalence of N-glycosylation between complementarity-determining regions (CDR) and the framework regions (FR), we did not include IGHV sequences lacking a fully designated V-D-J rearrangement (as per IMGT High V-quest).

Our intention was to evaluate the frequency of Ig sequences with N-glycosylation sites acquired as a result of SHM in autoimmune diseases. Hence, we also eliminated sequences

which were identical to germline variable heavy chain V and J genes (≤ 2 mutations in the V and J genes) as these may not have undergone SHM or their mutations could be attributed to the probability of Taq polymerase enzyme errors during PCR amplification. Naturally-occurring germline N-glycosylation sites were not counted in this analysis as we cannot evaluate the contribution of SHM to the selection of these Nglycs. Moreover, certain sequence datasets did not include the analyses of all IGHV genes and there would be a bias for or against the IGHV genes with natural-occurring sites.

The prediction of N-Glycosylation sites was carried out on the amino acid (aa) sequence of the IGHV-D-J region of all Ig sequences using the NetNglyc 1.0 Server (http://www.cbs.dtu.dk/services/NetNGlyc/) which predicts N-Glycosylation sites in the Asparagine-**X**aa-Serine/Threonine (N-X-S/T) motif, except when proline (P) is in position X or immediately after Ser/Thr, as this is known to eliminate glycosylation (6). We also excluded other motifs such as N-W(Tryptophan)-S, N-D(Aspartic acid)-S, N-E(Glutamic acid)-S and N-L(Leucine)-S, which were all reported to be poor oligosaccharide acceptor motifs (7). The criteria set for accepting prediction of N-glycosylation were Potential > 0.5 and Jury agreement ≥ 5/9 (8). Statistical analyses were performed using Pearson's Chi-square test (GraphPad Prism3.0; http:// www.graphpad.com/).

RESULTS

Increased prevalence of acquired N-glycosylation sites in Fab regions of Ig-producing cells from autoimmune diseases

The frequency of ac-Nglycs in the Fab-encoding regions of Ig repertoires and IgG sequences from normal, autoimmune and antigen-specific datsets are shown in Tables 1a and 1b, respectively. Overall prevalence of acquired N-glycosylation sites was significantly higher in the autoimmune repertoire when compared to those from normal tissues and antigen-specific datasets (Figure 1a). We wondered if the significant increase in N-glycosylation sites in the autoimmune group could be due to the fact all except one of its sequence datasets were from diseased tissues (non-blood), while a large number of sequences in the normal group were from the peripheral blood. To discount this, we compared the non-blood sequences of the normal and autoimmune group and found that the prevalence of acquired N-glycosylation sites was still significantly higher ($p=0.0395$) in autoimmune diseases (data not shown). When the datasets with isotype information were analyzed separately for IgM, IgG and IgA sequences, only IgG sequences from autoimmune diseases showed significantly higher prevalence of ac-Nglycs than IgGs from normal controls and antigen-specific datasets (Figure 1b).

We then analyzed for differences in the occurrence of ac-Nglycs in complementarity-determining regions (CDRs) versus framework-regions (FRs). The frequency of ac-Nglycs occurring in FRs of sequences from autoimmune diseases was significantly higher than those from normal controls and antigen-specific sequences (Figure 2a) and this difference was more stark among IgGs (Figure 2b). With respect to ac-Nglycs occurring in CDRs, sequences from autoimmune diseases generally showed a significantly higher prevalence than normal controls and anigen-specific sequences (Figure 2c). This pattern was consistent even among IgG sequences (Figure 2d).

A significant difference between Ig sequences from normal controls and antigen-specific datasets was only noted with respect to the prevalence of acquired N-glycosylation in IgG sequences (Figure 1b).

	Normals (n=2131)	ADs (n = 2809)	AgS (n = 817)
All Igs	64	274	23
	(n=527)	(n=375)	(n=521)
IgGs	1	50	8

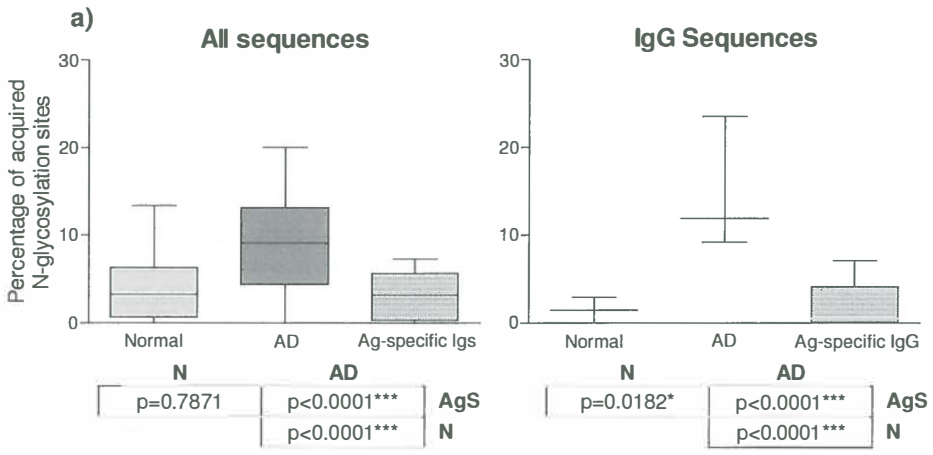

Figure 1: Prevalence of acquired N-glycosylation sites. The prevalence of acquired N-glycosylation sites is compared between autoimmune disorders (AD) and non-autoimmune or normal tissues (N) as well as antigen-specific sequences (AgS) in (a) all sequences from (regardless of isotype); (b) IgG sequences only. Differences between all combinations of groups are depicted using a two-point comparitive table under each graph with p-values from Pearson's chi-square analyses.

Acquired N-glycosylation motifs occur at identical Ig sites in different autoimmune diseases

It is apparent from Table 2 that the random processes of SHM in autoimmune diseases gave rise to the creation of new or acquired N-glycosylation sites at identical residues in different autoimmune disease datasets. For example, the acquired N-glycosylated site at residue 84 of IGHV3 genes were detected 61 times with 12 different motifs (NNT, NDT, NST, NNS, NIS, NYS, NTT, NSS, NKT, NHT, NGS and NYT). This cannot be attributed to identical or highly clonal Ig sequences as such sequences were excluded from our analyses. The consistent occurrence of identical N-glycosylation sites in different diseases suggests that the acquisition of N-glycosylation potential has some bearing on B cell selection and function.

Some of the potentially N-glycosylated residues detected in autoimmune diseases were also noted within the normal group and antigen-specific sequences, but their prevalence was far lesser than that seen in autoimmune diseases.

DISCUSSION

Previous studies on immunoglobulin variable (V) regions in follicular lymphomas (FL) revealed higher frequencies of acquired N-glycosylation sites compared to other B-cell malignancies and normal somatically mutated memory B-cells (3). The acquisition of N-glycosylation motifs by

All Sequences	Normals (n=2131)	ADs (n = 2809)	AgS (n = 817)
All Igs-FR	38	170	10
All Igs-CDR	26	104	13
IgGs	**(n=527)**	**(n=375)**	**(n=521)**
IgG-FR	0	27	3
IgG-CDR	1	23	5

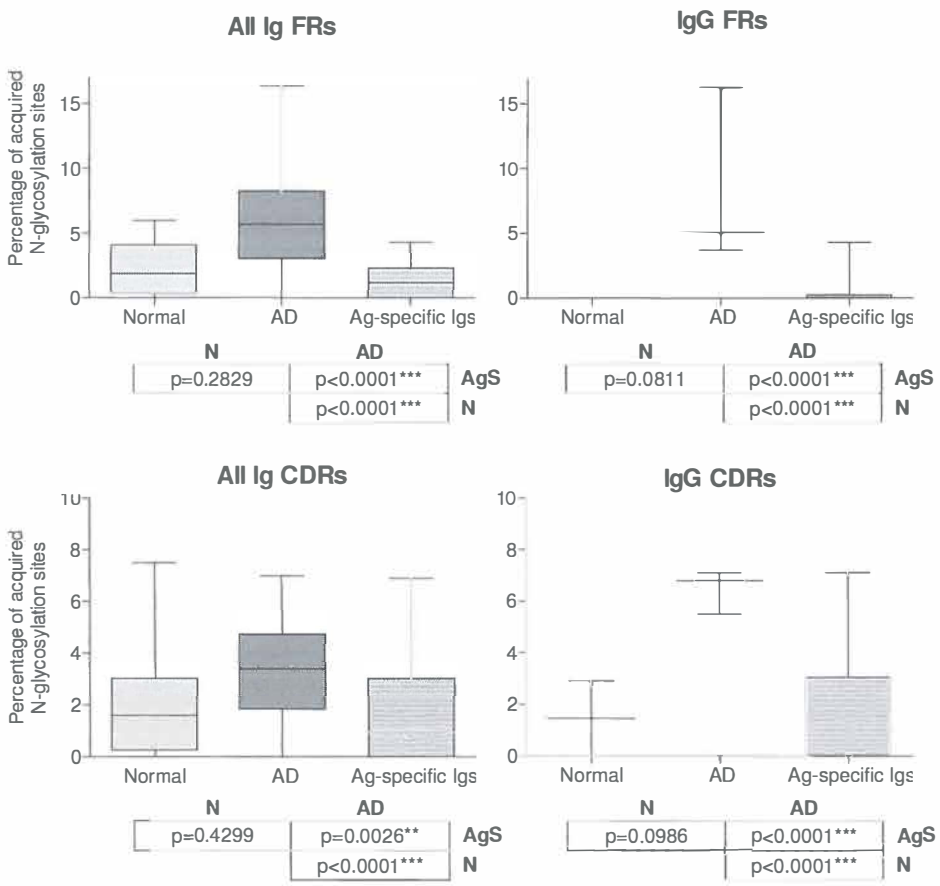

Figure 2: Distribution of acquired N-glycosylation sites. The prevalence of ac-Nglycs is compared between autoimmune diseases (AD) and non-autoimmune or normal tissues (N) as well as antigen-specific sequences (AgS) in (a) Framework regions (FRs) of all sequences (regardless of isotype) (b) Framework regions (FRs) of IgG sequences (c) Complementarity-determining regions (CDRs) of all sequences (regardless of isotype) (d) Complementarity-determining regions (CDRs) of IgG sequences. Differences between all combinations of groups are depicted using a two-point comparitive table under each graph with p-values from Pearson's chi-square analyses.

somatic hypermutation was demonstrated to confer a selective advantage to certain B cells in follicular lymphomas through a GC simulation model based on anti-hapten responses in mice (9). The relative lack of N-glycosylation motifs in the V-regions of normal memory B-cells, normal IgM and IgA plasma cells and non-follicular lymphoma B-cell malignancies suggests that the frequency of N-glycosylation sites is generally quite low in post-GC Ig-producing cells (3, 10, 11).

However, our study clearly indicates a significant selection of Ig-producing cells with acquired N-glycosylation motifs in autoimmune diseases compared to normal controls and antigen-specific B-cells. This selection is even more evident within IgG-producing cell populations. The relatively higher prevalence of acquired N-glycosylation motifs in autoimmune diseases within the FRs is in contrast to antigen-selected sequences, where most of the acquired N-glycosylation motifs occur in CDRs. This is also different to follicular lymphoma B cells, where the majority of ac-Nglycs (87%) are detected in CDRs (12). This strongly suggests that the increased frequency of ac-Nglycs in autoimmune diseases may be an alternative form of selection to classical antigen-specific binding or selection of follicular lymphoma B cells.

We {Hamza et al, submitted] and other studies (13) observed a significantly increased selection of B-cells exhibiting maintenance of structural integrity of Ig framework regions in autoimmune diseases compared to non-autoimmune samples. Interestingly, such an emphasis on IGHV framework regions is usually associated with B-cell superantigens, such as the Staphylococcal protein A (SpA), the endogenous human gut-associated sialoprotein, pFv and the HIV-1 envelope protein, gp120 (14). SpA interacts specifically with IGHV3 sequences between residues 75 and 84 of the FR3 region (15), with gp120 and pFv also sharing a similar affinity to same or nearby FR3 sites (14, 16). Such superantigen interactions with FRs ensure the availability of consistently conserved target regions on a large number of B cells and result in B-cell activation, differentiation and Ig secretion (14). These are examples of how B-cells can also be activated by interactions outside of conventional antigen-binding sites (CDRs).

Remarkably, 53.5% (76/142) of the acquired N-glycosylation motifs within IGHV3-expressing sequences from autoimmune repertoires (Table 2) were at the same locations where the IGHV3-specific superantigens such as SpA interact with B cell receptors (between FR3 residues 75 to 84). This suggests that such FR-associated acquired N-glycosylation motifs on immunoglobulins in autoimmune diseases may well mimic superantigen interaction via molecules such as lectins that bind to these sugar motifs. At the same time, we do not rule out the possibility that acquired N-glycosylation within CDRs may also activate B cells through lectin interactions, especially given that we detected multiple sequences from different autoimmune datasets with acquired N-glycosylation sites at identical residues (Table 2). This is noteworthy as we did not bias our data for or against N-glycosylated sequences by including identical and clonal sequences (≤ 2 unshared mutations) in our analysis. Moreover, the consistent pattern of acquired N-glycosylation sites in both CDRs and FRs also suggests that Fab interactions in autoimmune diseases are not subject to selection based on antigen-specificity.

The fact that the increased frequency of acquired N-glycosylation in autoimmune diseases is significantly associated to the IgG isotype also adds weight to the possibility of a functional outcome for acquired N-glycosylation within the Fab region. Moreover, the IgG phenotype signals the maturation of an antibody response and is essential for functioning within the effector arm of the immune response with regards to complement fixation. At this point, it is pertinent to note that SpA is also reported to interact with the constant region of IgG(14), thereby ensuring a simultaneous activation of not just IGHV3-expressing B cells (30-50% of total B cells), but also those B cells with specific effector functions (14).

Table 1: The distribution of acquired N-glycosylation sites

Dataset	Sample	No. of Patients	No. of mutated sequences (>2 mutations)	No. of n-Nglycs	No. of ac-Nglycs
NORMAL DATASETS					
(26)	BL, DNA, all Vh	4	116	0	6
(27, 28)	BL, CD19+ cells, DNA, all Vh	3	372	6	5
(29)	BL, RNA, all Vh, IgM	6	296	8	4
(30)	BL, CD19+ cells, RNA, all Vh, IgM	3	27	0	0
(31)	BL, RNA, all Vh, IgG	2	492	0	0
(32)	BL, RNA, all Vh, IgM+IgG+IgA	10	209	1	7
(33)	BM, RNA, Vh3, IgM	2	29	0	0
(34)	DD, RNA, all Vh, IgM+IgA	3	172	0	10
(35)	IN, RNA, all Vh, IgM+IgA	2	53	0	3
(36)	IL, RNA, all Vh, IgA	4	148	1	10
(37)	OM, RNA, Vh4 & Vh5, IgM+IgG+IgA	18	86	0	8
(38)	PT, RNA, all Vh, IgA	3	64	3	2
(24)	PT, RNA, Vh3, IgG+IgA	3	67	0	9
	TOTAL		2131	19	64
AUTOIMMUNE DISEASE DATASETS					
pSS (38)	PT, RNA, all Vh, IgA	4	89	1	3
pSS (24)	PT, RNA, Vh3, IgG+IgA	12	487	4	81
RA1 (39)	ST, RNA, all Vh, IgM+IgG+IgA	3	641	14	50
RA2 (40)	ST, DNA, all Vh	2	44		5
RA3 (41)	ST, RNA, all Vh	7	646	9	84
SLE1 (26)	BL, DNA, all Vh	7	341	10	18
SLE2 (42)	SP, DNA, all Vh	1	20		2
MS1 (43)	CSF, RNA, all Vh, IgM+IgG	2	88		8
MS2 (44)	CSF & BL, DNA, all Vh	*	112	3	10
ChD1 (45)	HRT, DNA, all Vh	3	15		2
ChD2 (45)	BM, DNA, all Vh	1	55		11
AS (46)	SM, DNA, all Vh	1	29		1
WG (47, 48)	NT, DNA, all Vh	6	242		0
	TOTAL		2809	41	274

Total frequency of ac-Nglycs (%)	No. of ac-Nglycs / region (%)						Total No. of FR ac-Nglycs	Total No. of CDR ac-Nglycs	% of FR ac-Nglycs	% of CDR ac-Nglycs
	FR1	CDR1	FR2	CDR2	FR3	CDR3				
5,2	1				3	2	4	2	3,4	1,7
1,3		1		1	3		3	2	0,8	0,5
1,4				1	2	1	2	2	0,7	0,7
0,0							0	0	0,0	0,0
0,0							0	0	0,0	0,0
3,3	1			1	5		6	1	2,9	0,5
0,0							0	0	0,0	0,0
5,8		1		3	6		6	4	3,5	2,3
5,7				2	1		1	2	1,9	3,8
6,8	2	1		1	5	1	7	3	4,7	2,0
9,3		1		2	4	1	4	4	4,7	4,7
3,1	1					1	1	1	1,6	1,6
13,4	1	3			3	2	4	5	6,0	7,5
	6	7	0	11	32	8	38	26		
3,4		0		1	2	0	2	1	2,2	1,1
16,6		20	1	11	46	3	47	34	9,7	7,0
7,8	5	17	1	3	16	8	22	28	3,4	4,4
11,4		2			3		3	2	6,8	4,5
13,0	10	7		11	52	3	63	21	9,8	3,3
5,3		8		1	9		9	9	2,6	2,6
10,0		1			1		1	1	5,0	5,0
9,1		2			5	1	5	3	5,7	3,4
8.9		1	1	1	6	1	7	3	6,3	2,7
13,3				1	1		1	1	6,7	6,7
20,0	4	1	3	1	5	--	9	2	16,4	3,6
3,4	--	--	--	--	1	--	1	0	3,4	0,0
0,0	--	--	--	--	--	--	0	0	0,0	0,0
	19	59	3	30	147	16	170	104		

Dataset	Sample	No. of Patients	No. of mutated sequences (>2 mutations)	No. of n-Nglycs	No. of ac-Nglycs
ANTIGEN SPECIFIC DATASETS					
(49)	H1N1 [r] , RNA All Vh, IgA+IgG	8	55		4
(50, 51)	Antigen-specific [ab/cs/h/pl], RNA+DNA, All Vh	*	80		4
(23, 51, 52)	Anti-HibCP [v] (16 individuals, All Vh, IgM+IgG+IgA	16	58		4
(53)	Anti-TT1 [v/pl] , RNA, All Vh	2	53		1
(54)	Anti-TT2 [v/cs], RNA, Vh3, IgG	4	81		2
(50, 55)	Anti-CMV [cs], RNA, Vh3, IgG	3	23		0
(56)	Rotavirus1 [h], RNA, All Vh	8	48		3
(57)	Rotavirus2 [cs], RNA, All Vh, IgG	10	186		1
(58)	Rotavirus3 [cs], RNA, All Vh, IgG	11	47		2
(59-61)	Strept. Pneum [v], RNA, All Vh	15	53		2
(62)	Anti-PPS [v/cs] , RNA, All Vh, IgG	40	100		0
(63, 64)	StaphA [ab/pl], RNA, Vh1/3/4, IgM+IgG	4	33		0
(49)	TOTAL		817		23

Prevalence of acquired N-glycosylation motifs in (a) all Ig sequences from normal controls, autoimmune disease patients and from antigen-specific sequence datasets; (b) IgG sequences from normal controls, autoimmune disease patients and from antigen-specific sequence datasets. References provided indicate the published studies from which the sequence datasets were obtained. Abbreviations used are as follows: pSS - Sjogren's syndrome, RA - rheumatoid arthritis, SLE - systemic Lupus erythrematosus, MS - multiple sclerosis, ChD - Chaga's disease, AS - ankylosing spondylitis, WG - Wegener's granulomatosis, BL - blood, BM - bone marrow, DD - duodenum, IN - intestine, IL - ileum, OM - omentum, PT - parotid salivary gland, ST - synovial tissue, SP - spleen, CSF - cerebrospinal fluid, HRT - heart tissue, SM - synovial membrane, TH - thymus, NT - nasal tissue, [r] - reperotire study, [ab] - antigen - binding B cells, [cs] - B cell sorted by antigen - specific binding, [h] - B cell hybridoma secreting antigen - binding antibodies, [pl] - antigen - binding antibodies from expressed phage library, [v] - antigen binding B cells after vaccination.

Total frequency of ac-Nglycs (%)	No. of ac-Nglycs / region (%)						Total No. of FR ac-Nglycs	Total No. of CDR ac-Nglycs	% of FR ac-Nglycs	% of CDR ac-Nglycs
	FR1	CDR1	FR2	CDR2	FR3	CDR3				
7.3				2	2	0	2	2	3,6	3,6
5.0		1		1	2	0	2	2	2,5	2,5
6.9		1		1		2	0	4	0,0	6,9
1.9					1		1	0	1,9	0,0
2.5		2					0	2	0,0	2,5
0.0							0	0	0,0	0,0
6.3					1	2	1	2	2,1	4,2
0.5					1		1	0	0,5	0,0
4.7					2		2		4,3	0,0
3.8		1			1		1	1	1,9	1,9
0.0									0,0	0,0
0							0	0	0,0	0,0
	0	5	0	4	10	4	10	13		

Dataset	Sample	No. of Patients	No. of mutated sequences (>2 mutations)	No. of ac-Nglycs	Total frequency of ac-Nglycs (%)
NORMAL DATASETS					
(31)	BL, RNA, all Vh, IgG	2	492	0	0
(24)	PT, RNA, Vh3, IgG	3	35	1	2.9
	TOTAL		527	1	
AUTOIMMUNE DISEASE DATASETS					
pSS (24)	PT, RNA, Vh3, IgG	12	98	23	23,5
RA1 (39)	ST, RNA, all Vh, IgG	3	218	20	9,2
MS1 (43)	CSF, RNA, all Vh, IgG		59	7	11,9
	TOTAL		375	50	
ANTIGEN-SPECIFIC DATASETS					
(50, 51)	Various antigen-specific Igs[h], RNA+DNA, All Vh, IgG	*	28	2	7.1
(23)	Anti-HibCP [v], RNA, All Vh, IgG	1	15	0	0.0
(50, 55)	Anti-CMV [h], RNA, Vh3, IgG	3	23	0	0.0
(54)	Anti-TT2 [v/cs], RNA, Vh3, IgG	4	81	2	2.5
(57)	Rotavirus2 [cs], RNA, All Vh, IgG	10	186	1	0.5
(58)	Rotavirus3 [cs], RNA, All Vh, IgG	11	47	2	4.7
(62)	Anti-PPS [v/cs] , RNA, All Vh, IgG	40	100	0	0.0
(59, 60)	Anti-SP [v], RNA, All Vh, IgG	15	28	1	3.6
(64)	StaphA [pl], RNA, Vh1/3/4, IgG	1	13	0	0
	TOTAL		521	8	

Prevalence of acquired N-glycosylation motifs in (a) all Ig sequences from normal controls, autoimmune disease patients and from antigen-specific sequence datasets; (b) IgG sequences from normal controls, autoimmune disease patients and from antigen-specific sequence datasets. References provided indicate the published studies from which the sequence datasets were obtained. Abbreviations used are as follows: pSS - Sjogren's syndrome, RA - rheumatoid arthritis, SLE - systemic Lupus erythrematosus, MS - multiple sclerosis, ChD - Chaga's disease, AS - ankylosing spondylitis, WG - Wegener's granulomatosis, BL - blood, BM - bone marrow, DD - duodenum, IN - intestine, IL - ileum, OM - omentum, PT - parotid salivary gland, ST - synovial tissue, SP - spleen, CSF - cerebrospinal fluid, HRT - heart tissue, SM - synovial membrane, TH - thymus, NT - nasal tissue, [r] - reperotire study, [ab] - antigen - binding B cells, [cs] - B cell sorted by antigen - specific binding, [h] - B cell hybridoma secreting antigen - binding antibodies, [pl] - antigen - binding antibodies from expressed phage library, [v] - antigen binding B cells after vaccination.

No. of ac-Nglycs / region (%)						Total No. of FR ac-Nglycs (%)	Total No. of CDR ac-Nglycs (%)	% of FR ac-Nglycs	% of CDR ac-Nglycs
FR1	CDR1	FR2	CDR2	FR3	CDR3				
						0	0	0,0	0,0
			1			0	1	0,0	2,9
0	0	0	1	0	0	0	1		
--	--	1	4	15	3	16	7	16,3	7,1
1	9	--	--	7	3	8	12	3,7	5,5
--	2	--	--	3	2	3	4	5,1	6,8
1	11	1	4	25	8	27	23		
	1		1			0	2	0,0	7,1
						0	0	0,0	0,0
						0	0	0,0	0,0
			2			0	2	0,0	2,5
				1		1	0	0,5	0,0
				2		2		4,3	0,0
								0,0	0,0
	1					0	1	0,0	3,6
						0	0	0,0	0,0
0	2	0	3	3	0	3	5		

Table 2: Acquired N-glycosylation site residues and motifs

IGHV gene	No. of ac-Nglycs	Acquired N-glycosylation site residues and amino acid (aa) motifs										
		FR1		CDR1		FR2		CDR2		FR3		CDR3
		aa	Motif	aa	Motif	aa	Motif	aa	Motif	aa	Motif	Motif
NORMAL TISSUES (n = 2131)												
Vh1	6			29	NFS			64	NTT	81	NTS, NSS	NYS, NGT
Vh2	2	24	NFS							68	NTS	
Vh3	30	26	NSS	29 35	NVS, NFS(2) NSS			57 62 63 64	NNT, NYS NGS, NYT NNT, NRT NKS	77 84 97	NIS(3) NNT(9), NGS, NKS NDT	NVS, NVT, NRT
Vh4	16	24	NVS(3)	31	NSS			57	NYS(2)	68 90	NSS(2), NAS NLT(6), NVT	
Vh5	10	20	NIS	35	NTS			64	NTT	75 77 85 95	NVT NIS NTT NAS	NVS, NTT, NYS
AUTOIMMUNE DISEASES (n = 2536)												
Vh1	47	20 23	NVS(7) NVS	29	NFT (3)	51	NFT	63 64 66	NIT, NST NTS NYS, NYT	66 77 81 82 91 93 97	NYT(3), NYS NMT, NLT(3), NFT, NIT(2) NQS, NTS(4), NIS NST NDT NLT NDT(3)	NGT(2), NST, NIT, NET
Vh2	18			27 31 - 34	NFS NTT(2), NAS, NTS(2) NAS			64	NKS	68 77	NIS, NTS NIS, NIT(2)	NGS(2), NGT, NSS, NVT

Vh3	142	26	NKT	27 29 . . 30 35 36	NFT NFT(5), NFS(15), NSS, NIS, NLT, NMT NSS NVS, NRT NYS(2)	55 .	NIS, NGS	57 59 62 63 . . 64 65	NTS NGS(4), NGT NGT NET(2), NQT, NYT(2), NTT, NRT, NVT, NYS, NNT NTT, NKS NYT	66 77 81 82 84 . . 90 93 97	NYT NIS(8), NVS, NIT NNS(3), NTS NST NNT(42), NDT(3), NST(3), NNS, NIS, NYS(2), NTT, NSS(2), NKT(2), NHT, NGS(2), NYT NLT NLT(7) NDT(2)	NFT, NFS, NGT, NNT, NVT, NYS
Vh4	46	15 22 . 24	NFS NCS, NVS(2) NVS(4)	27 31 34	NGS, NGT NSS(2) NVS			57 59	NYS(2), NRS NGS	66 68 77 81 90 93 96	NSS NAS(4), NSS (5) NIS(2) NTS NLT(10), NIS, NVT, NMT NLT NVT	
Vh5	13	24	NGS	29 35	NFT(7) NST					68 81 82 84	NTS, NRS NKS NST NTT	
Vh6	7			29 34 35	NIS, NFS NNT NRS(2), NNT					81	NTS	
Vh7	1	20	NIS									
ANTIGEN-SPECIFIC SEQUENCES (n = 817)												
Vh1	1									85	NTT	
Vh2	2									68	NTS(2)	
Vh3	14			27 29 36	NGS NFS(2) NNT, NSS			63	NKT, NGT	84 93	NNT(3) NLT	NFT, NGS, NYS
Vh4	6							59	NGS (2)	68 90	NTS, NSS NVS	NGT

The residue (aa) and motif of the acquired N-glycosylation site in Ig sequences from normal tissues, autoimmune diseases and antigen-specific sequences are shown, alongwith their occurrence in different IGHV genes and their situation in CDRs or FRs.

We observed low frequencies of acquired N-glycosylation sites in the AS dataset and no acquired N-glycosylation sites in two separate datasets from WG patients. This could be due to the greater presence of non-IgG isotypes, particularly IgM, which is generally less mutated than IgG and hence, less likely to acquire N-glycosylation sites through SHM. Alternatively, it may also indicate differences in selection pressures among systemic autoimmune diseases and could form the basis for a re-classification of these diseases.

We did observe a few naturally-occurring N-glycosylation sites in sequences expressing the IGHV1-8 gene in both autoimmune and normal samples (Tables 2a and 2b), whereas natural sites in the IGHV-4 gene were never detected. However, these sites were not included in our statistical analyses as we cannot evaluate their contribution to SHM-based selection. Moreover, we did not detect any IgG sequences with the IGHV1-8 naturally-occurring sites within Ig sequences from normal tissues, autoimmune diseases or antigen-specific sequences. This suggests that the IGHV1-8 natural N-glycosylation site is neutral and its loss probably has no effect on B cell selection.

As previously shown by Coelho et al (17), the presence of glycosylation moieties in the conserved sites of Ig variable regions may enable B-cells to keep the impetus for B-cell activation and proliferation by interacting with lectin-expressing cells such as macrophages or dendritic cells or perhaps even T cells (18) and NK cells (19). Hence, we propose a novel hypothesis that the significant presence of Ig-producing cells with potential acquired N-glycosylation sites in certain systemic autoimmune diseases may be indicative of an unconventional selection pressure that mimics superantigen-like interactions on B cells and enables B cells with glycosylated immunoglobulins to engage, survive and persist within autoimmune lesions.

There is some discussion in the scientific community with regards to the use of superantigen-based therapies in the treatment of systemic autoimmune disorders (20, 21). The main evidence in support of this comes from the use of SpA therapy in a murine experimental model for SLE which succeeded in limiting disease progression (22). Our hypothesis of an altered B cell selection in systemic autoimmune diseases homologous to B cell and superantigen interactions may offer a cautionary insight towards the development of such therapies in humans(23). It is important to note that while B cell superantigens are specific for certain B cell subsets and those that express particular genes, we did not observe any such specificity with regards to the prevalence of acquired N-glycosylation sites in autoimmune diseases. Moreover, superantigen-based therapies are essentially intended to be B cell targeting strategies and are therefore similar to B cell-depletion therapies such as rituximab (RTX). Our group has already shown in the context of pSS that while RTX does result in clinical relief from disease symptoms, the relief is transient and treated patients suffer disease relapses [Hamza et al, submitted]. Parallel to this, we also showed that despite the widespread depletion of B cells in the peripheral circulation, the prevalence of acquired N-glycosylation sites in Ig sequences from diseased salivary glands of pSS patients was not altered even after RTX. This implies that B cell targeting strategies in systemic autimmune diseases only succeeds in diminishing the effector B cell numbers and does not, figuratively, switch-off the underlying pathological signaling mechanisms. Hence, we speculate that our findings may also provide a strong rationale for the development of alternative therapies that seek to modulate systemic autoimmune disease through the use of anti-glycosylating compounds (24) or glycans that can competitively bind to lectins on dendritic cells thereby interfering with the recruitment of a deleterious (auto)immune response (25).

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6

EVIDENCE FOR ALTERED SELECTION PRESSURES ON B CELLS IN AUTOIMMUNE DISEASES

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Manuscript in preparation

ABSTRACT

Objectives: Our previous studies on the immunoglobulin variable heavy chain (IGHV) repertoires indicated that prevalence of acquired N-glycosylation motifs (ac-Nglycs) in autoimmune diseases differed from that seen in normal or non-autoimmune (control) repertoires and classical antigen-driven repertoire. Our main objective was to computationally evaluate the selection pressures in autoimmune diseases, controls and classical antigen-specific repertoires and correlate these results with the increased prevalence of ac-Nglycs in IGHV repertoires from autoimmune diseases. This would enable a better understanding of the selection pressures shaping the autoimmune repertoire.

Methods: We analyzed the selection pressures acting on the IGHV sequences from our previous studies in pSS and other published datasets from multiple sclerosis (MS), RA, SLE, Chagas's Disease (ChD), Ankylosing spondylitis (AS) and Wegener's granulomatosus (WG). As controls, we collected published datasets of IGHV sequences derived from various control tissues and antigen-specific B cells from vaccination or infection studies. All sequences were analyzed for selection pressures using a new computational method known as Bayesian estimation of Antigen-driven SELectIoN (BASELINe).

Results: IgG sequences from autoimmune diseases showed no positive selection in their CDRs, while exhibiting significantly high conservation of structure in their FWRs compared to IgG sequences in control and antigen-driven repertoires. We also observed that the selection pressures in all IgG sequences and more particularly, IgG sequences with ac-Nglycs within the autoimmune repertoire, were similar to the selection pressures observed in IgG sequences from B cells specific for the superantigen Staphylococcal protein A (SpA). IgG sequences with ac-Nglycs from autoimmune diseases also shared some similarities in selection pressures with IgG sequences from B cells specific for the pneumococcal polysaccharide antigen, PPS which is known to engender immune responses through its interaction with lectin-expressing cells via a T-independent process.

Conclusions: This study suggests that the autoimmune repertoire and particularly the IgG-producing populations in autoimmune diseases are probably not selected by classical (auto)antigen-driven selection but exhibit selection pressures that are more similar to IgGs from superantigen-specific immune responses. Our observations also provided evidence for the hypothesis that in the presence of altered selection pressures, IgG-producing B cells with ac-Nglycs in autoimmune diseases could possibly add a second level of selection through binding to lectins of the innate immune system.

INTRODUCTION

Autoimmune diseases are primarily characterized by the excessive production of antibodies that bind self-antigens and are accompanied by a cascade of pathological events which result in the immune system being harnessed to destroy specific tissues or glands of the body. Under normal conditions, B cells follow tightly regulated developmental and differentiation pathways interspersed with many check points to prevent the distraction of a homeostatic immune response into autoimmunity or uncontrolled B cell hyperactivity. In autoimmune disorders, it is apparent that at some point in the B cell differentiation pathway, the usual checkpoints aimed at eliminating self-reactive B cells, have failed. However, the precise etiology behind autoimmune diseases, remains a mystery.

Most autoimmune disorders are accompanied by B cell hyperactivation and proliferation, possibly culminating in autoantibody production. Since each B cell codes for a unique B cell receptor (BCR), we analyzed immunoglobulin heavy chain variable genes (IGHV) to study the selection pressures on B cells in autoimmune disorders which likely include auto-reactive B cell populations. The critical tools in the study of selection pressures are computational immunology programs whose statistical algorithms are designed to predict the type and extent of selection pressures that shape the B cell repertoire on the basis of mutation patterns.

Within immunoglobulin variable region structure, framework-regions (FWRs) of both heavy and light chains serve as the scaffolding on which complementarity-determining region (CDR) loops are optimized through somatic hypermutation (SHM) (1). FWRs in IGHV genes are generally well conserved regions primarily because they are less prone to be selected for non-conservative mutations compared to CDRs (2) . In a previous study using the computational tool, the Focused-z test (3); we observed that immunoglobulin sequences from diseased salivary glands of patients with pSS, a classic autoimmune disorder, demonstrated significantly increased conservation of framework-region (FWR) structure compared to non-pSS control patients despite being heavily mutated (4).

Such an emphasis on framework-region maintenance was reminiscent of BCR-superantigen interactions that characteristically occur at conserved FWRs (5, 6). For a B cell-superantigen, this ability ensures the availability of binding targets minimally influenced by the unpredictability of genetic recombination (as seen in CDR3) or the hypervariability introduced by SHM (mostly focused on CDRs). This is best exemplified by the superantigen Staphylococcal protein A (SpA), whch interacts between residues 75 to 84 within the FWR3 domain of the immunoglobulin heavy chain (IGHV)-3 gene family, that is expressed by 30-50% of the total B cell repertoire in healthy individuals (5, 7). Thus, BCR-superantigen interactions indicate two important points; one, since interactions at these sites by superantigens lead to B cell activation, proliferation, differentiation and immunoglobulin secretion (5), it is conceivable that a similar engagement of the BCR by other moieties may result in the same respone. The second point is that the emphasis on maintaining overall structure of FWRs outside of classical antigen-binding sites (CDRs) could also be a sign of B cell selection.

In pSS patients, we also noted a surprisingly low evidence for strong antigenic selection in IGHV sequences. This seemed to contradict the widely-held belief that B cells are hyperactivated in autoimmune disorders due to (auto)antigen-driven selection (8-12). Consequently, in our exploration for alternative selection mechanisms, we observed a significantly increased prevalence of acquired N-glycosylation sites in the IGHV regions of IgG sequences obtained from pSS patients compared to non-pSS controls. These acquired N-glycosylation sites were novel

motifs created during SHM and persisted even after B cell depletion treatment of pSS patients with rituximab (4). Moreover, 60% of the acquired N-glycosylation motifs were noted at sites identical to those interacting with SpA (residues 75 to 84). This increased presence of acquired N-glycosylation sites was not restricted to pSS, but could also be noted in other autoimmune diseases such as rheumatoid arthritis (RA), multiple sclerosis (MS), lupus erthrematosus (SLE) and Chagas's Disease (ChD). Furthermore, the prevalence of these sites in autoimmune diseases was significantly greater than that observed in normal or non-autoimmune tissues and antigen-specific datasets (13). Again, we observed that a large proportion of the acquired N-glycosylation motifs within IGHV3-expressing sequences in autoimmune disorders consistently occurred at residues 75 to 84 of FWR3, the exact same sites of SpA-BCR interactions (7).

This lead us to hypothesize that sugar moieties on BCRs could possibly mimic BCR-superantigen interactions in its independency from antigen-driven selection of B cells. We speculated that increased acquisition of N-glycosylation motifs may represent a form of unconventional selection pressure on B cell repertoires in autoimmune diseases. Hence, our aim in this study was to to explore the correlations between selection pressures and prevalence of acquired N-glycosylation sites in autoimmune diseases.

METHODS

We analyzed IGHV sequence data from our previous studies in pSS and other published datasets from multiple sclerosis (MS), rheumatoid arthritis (RA), lupus erthrematosus (SLE), Chagas's Disease (ChD), ankylosing spondylitis (AS) and granulomatosis with polyangiitis (GPA; formerly known as Wegener's granulomatosus). As controls, we collected published datasets of IGHV sequences derived from various normal and non-autoimmune (control) tissues such as blood, bone marrow, duodenum, ileum, omentum and parotid salivary glands. In this study, we also included sequences derived from antigen-specific B cells or B cell repertoires from vaccination and infection studies (Tables 1a and 1b) against antigens such as H1N1, haemophilus influenza B capsular poysaccharide (HibCP) with protein adjuvant, tetanus toxoid (TT), cytomegalovirus (CMV) and rotavirus. Moreover, we included IGHV sequences derived from B cells specific for SpA and pneumococcal polysaccharide antigen (PPS) as positive controls for selection pressures due to superantigen interaction and polysaccharide antigenic stimulation, respectively.

All IGHV sequences were compared with the international ImMunoGeneTics (IMGT) databases of Ig germline sequences by only using the high-throughput tool, High V-Quest (14), since the IGHV gene identification was different when the low-throughput IMGT V-quest was used, particularly in sequences with ambiguous nucleotide reads. All sequences that were unproductive, non-Ig and identical or redundant sequences were eliminated from our analyses. Clonal sequences with ≤ 2 unshared mutations were also excluded if they expressed identical isotypes. As we wanted to compare the selection pressures influencing complementarity-determining regions (CDR) and framework regions (FWR), we did not include IGHV sequences lacking a full V-region (as per IMGT High V-quest).

Analysis of selection pressures

Selection pressure analysis was carried out with an online program known as the Bayesian estimation of Antigen-driven SELectIoN (BASELINe) (15). BASELINe provides a more quantitative and visual method to analyze selection. It incorporates statistical algorithms designed to predict the type and extent of selection pressures which shape the Ig repertoire

on the basis of mutation patterns. Affinity maturation is a process by which B cells accumulate somatic mutations within their immunoglobulin genes after which they are selected based on their affinity to antigens. The distribution of replacement versus silent (R/S) mutations, in both CDRs and FWRs is counted separately and compared against the expected frequency under the hypothesis of no selection as defined by germline genes. The highlights of BASELINe are its ability to aggregate the selection strengths of different sequences within a single experimental group and also compare the selection pressures between different experimental groups (15). Only mutated sequences (≥ 2 mutations) were included in this study.

RESULTS

All datasets used in this study are as shown in Table 1. We analyzed 2809 IGHV sequences from autoimmune diseases, 2131 from normal or non-autoimmune controls and 817 from antigen-specific datasets. Among the antigen-specific sequences, we separated out those sequences coding for anti-SpA and anti-PPS antibodies from the rest of the sequences and used them as positive controls for superantigen interaction and polysaccharide antigen-specific antibody response, respectively

Emphasis on conservation of FWRs rather than antigen selection in IgG from autoimmune disorders

IGHV sequences from autoimmune diseases showed almost no positive selection in their CDRs compared to both controls and antigen-driven repertoires (Figure 1). At the same time, FWRs in IGHV sequences from autoimmune diseases demonstrated the influence of significantly high negative selection pressures compared to control and antigen-driven repertoires. Among IgM, IgA and IgG isotypes, only IgG sequences from autoimmune diseases demonstrated a similar pattern of significant differences compared to controls and antigen-driven repertoires (Figure 2). This indicated that selection pressures in autoimmune diseases are associated with a particular IgG-specific immune response.

B cell selection pressures in autoimmune diseases show similarities with response to superantigens

The comparison of sequences from anti-SpA IgG-producing cells and classical antigen-driven responses indicated that conservation of FWR and the extent of negative selection in CDRs were significantly higher in anti-SpA IgG compared to antigen-driven repertoires (Figure 2). In contrast, anti-SpA IgM showed signs of significant positive antigen selection in CDRs that was similar to antigen-driven sequences, while the frame-work regions in anti-SpA IgM showed almost no sign of selection (data not shown).

Comparison of IgG sequences from autoimmune disorders, control tissues and classical antigen-driven datasets against anti-SpA IgG (Figure 2) confirmed that conservation of FWRs was greatest in anti-SpA IgG, with IgG from autoimmune diseases showing a similar selection. In contrast to this, the classical antigen-driven datasets and the control group showed significantly less conservation of FWRs. With regards to the CDRs of IgG sequences from the same groups, we observed that only IgG sequences from the control repertoire and antigen-driven datasets showed significant positive selection. On the other hand, IgG sequences from autoimmune diseases exhibited CDRs that were under negative selection pressures, although not to the extent seen in anti-Staph A IgG.

Table 1: IGHV sequence datasets analyzed

Dataset	Sample	No. of Patients	No. of mutated sequences (>2 mutations)
CONTROL REPERTOIRES			
(21)	BL, DNA, all Vh	4	116
(22, 23)	BL, CD19+ cells, DNA, all Vh	3	372
(24)	BL, RNA, all Vh, IgM	6	296
(25)	BL, CD19+ cells, RNA, all Vh, IgM	3	27
(26)	BL, RNA, all Vh, IgG	2	492
(27)	BL, RNA, all Vh, IgM+IgG+IgA	10	209
(28)	BM, RNA, Vh3, IgM	2	29
(29)	DD, RNA, all Vh, IgM+IgA	3	172
(30)	IN, RNA, all Vh, IgM+IgA	2	53
(31)	IL, RNA, all Vh, IgA	4	148
(32)	OM, RNA, Vh4 & Vh5, IgM+IgG+IgA	18	86
(33)	PT, RNA, all Vh, IgA	3	64
(34)	PT, RNA, Vh3, IgG+IgA	3	67
TOTAL			2131
AUTOIMMUNE DISEASE REPERTOIRES			
pSS (33)	PT, RNA, all Vh, IgA	4	89
pSS (34)	PT, RNA, Vh3, IgG+IgA	12	487
RA1 (35)	ST, RNA, all Vh, IgM+IgG+IgA	3	641
RA2 (36)	ST, DNA, all Vh	2	44
RA3 (37)	ST, RNA, all Vh	7	646
SLE1 (21)	BL, DNA, all Vh	7	341
SLE2 (38)	SP, DNA, all Vh	1	20
MS1 (39)	CSF, RNA, all Vh, IgM+IgG	2	88
MS2 (40)	CSF & BL, DNA, all Vh	*	112
ChD1 (41)	HRT, DNA, all Vh	3	15
ChD2 (41)	BM, DNA, all Vh	1	55
AS (42)	SM, DNA, all Vh	1	29
WG (43, 44)	NT, DNA, all Vh	6	242
TOTAL			2809
ANTIGEN-SPECIFIC REPERTOIRES			
(45)	H1N1 [r] , RNA All Vh, IgA+IgG	8	55
(46-48)	Anti-HibCP [v] (16 individuals, All Vh, IgM+IgG+IgA	16	58
(49)	Anti-TT1 [v/pl] , RNA, All Vh	2	53
(50)	Anti-TT2 [v/cs], RNA, Vh3, IgG	4	81
(51, 52)	Anti-CMV [cs], RNA, Vh3, IgG	3	23
(53)	Rotavirus1 [h], RNA, All Vh	8	48
(54)	Rotavirus2 [cs], RNA, All Vh, IgG	10	186

Dataset	Sample	No. of Patients	No. of mutated sequences (>2 mutations)
(55)	Rotavirus3 [cs], RNA, All Vh, IgG	11	47
Polysaccharide antigen-specifc repertoires			
(56-58)	Strept. Pneum [v], RNA, All Vh	15	53
(59)	Anti-PPS [v/cs] , RNA, All Vh, IgG	40	100
Superantigen-specific repertoires			
(60, 61)	StaphA [ab/pl], RNA, Vh1/3/4, IgM+IgG	4	33
(45)			
TOTAL			817
CONTROL REPERTOIRES			
(26)	BL, RNA, all Vh, IgG	2	492
(4)	PT, RNA, Vh3, IgG	3	35
TOTAL			527
AUTOIMMUNE DISEASE REPERTOIRES			
pSS (4)	PT, RNA, Vh3, IgG	12	98
RA1 (35)	ST, RNA, all Vh, IgG	3	218
MS1 (39)	CSF, RNA, all Vh, IgG		59
TOTAL			375
ANTIGEN-SPECIFIC REPERTOIRES			
(48)	Anti-HibCP [v], RNA, All Vh, IgG	1	15
(51, 52)	Anti-CMV [h], RNA, Vh3, IgG	3	23
(50)	Anti-TT2 [v/cs], RNA, Vh3, IgG	4	81
(54)	Rotavirus2 [cs], RNA, All Vh, IgG	10	186
(55)	Rotavirus3 [cs], RNA, All Vh, IgG	11	47
Polysaccharide antigen-specifc repertoires			
(59)	Anti-PPS [v/cs] , RNA, All Vh, IgG	40	100
(56, 57)	Anti-SP [v], RNA, All Vh, IgG	15	28
Superantigen-specifc repertoires			
(61)	StaphA [pl], RNA, Vh1/3/4, IgG	1	13
TOTAL			521

(a) All Ig sequences from normal controls, autoimmune disease patients and from antigenspecific sequence datasets; (b) IgG sequences from normal controls, autoimmune disease patients and from antigen-specific sequence datasets. References provided indicate the published studies from which the sequence datasets were obtained. Abbreviations used are as follows: pSS - Sjogren's syndrome, RA - rheumatoid arthritis, SLE - systemic Lupus erythrematosus, MS - multiple sclerosis, ChD - Chaga's disease, AS - ankylosing spondylitis, WG - Wegener's granulomatosis, BL - blood, BM - bone marrow, DD - duodenum, IN - intestine, IL - ileum, OM - omentum, PT - parotid salivary gland, ST - synovial tissue, SP - spleen, CSF - cerebrospinal fluid, HRT - heart tissue, SM - synovial membrane, TH - thymus, NT - nasal tissue, [r] - reperotire study, [ab] - antigen - binding B cells, [cs] - B cell sorted by antigen - specific binding, [h] - B cell hybridoma secreting antigen - binding antibodies, [pl] - antigen - binding antibodies from expressed phage library, [v] - antigen binding B cells after vaccination.

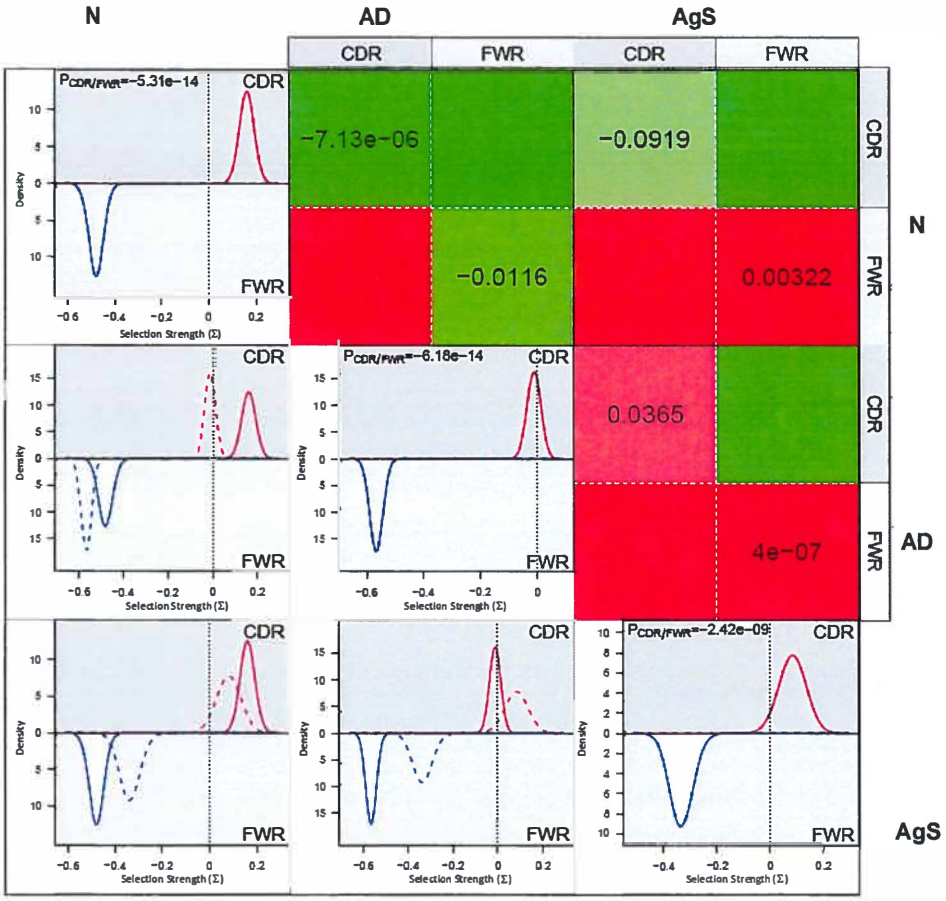

Figure 1: Comparison of selection pressures in all sequences regardless of isotype. The selection pressures in all groups were compared in a two-point table format with the numbers indicating statistical difference between parameters in a particular column over that in the corresponding row. Negative sign and green colour indicate negative selection pressures, while red clour indicates positive selection . The strength of the selection pressure is also indicated with varying intensities of the colour codes. Abbreviations used are as follows: N - normal datasets, AD - autoimmune disease datasets, AgS - classical antigen-driven repertoires, CDR - complementarity-determining region, FWR - framework region.

Correlation between acquisition of N-glycosylation sites and selection pressures

In our previous study, a large number of the acquired N-glycosylation motifs within IGHV3-expressing sequences (~53%) in autoimmune disorders consistently occurred at residues 75 to 84 of FWR3, the exact same sites of SpA-BCR interactions [Chapter 5]. Hence, we included anti-SpA IgG as a positive control for selection pressures in BCR-superantigen interactions in order to compare the selection pressures created by the acquisition of N-glycosylation motifs.

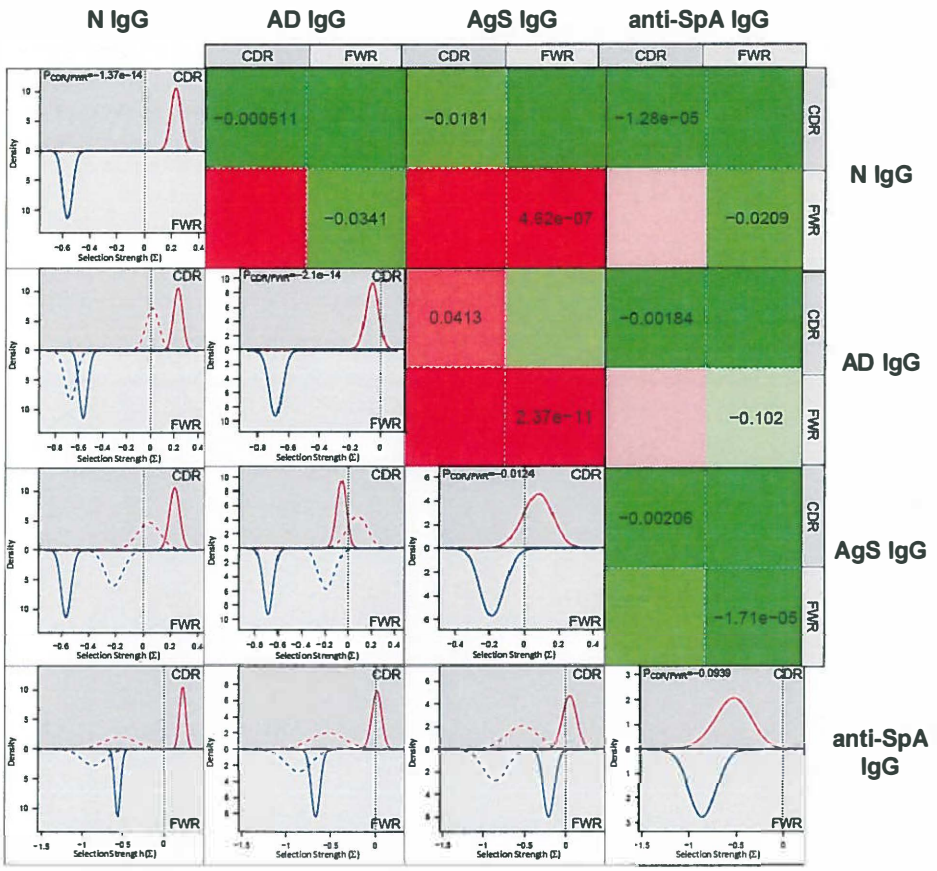

Figure 2: Comparison of selection pressures in IgG sequences. The selection pressures in IgG sequences were compared between different groups in a two-point table format with the numbers indicating statistical difference between parameters in a particular column over that in the corresponding row. Negative sign and green colour indicate negative selection pressures, while red clour indicates positive selection . The strength of the selection pressure is also indicated with varying intensities of the colour codes. Abbreviations used are as follows: N - normal datasets, AD - autoimmune disease datasets, AgS - classical antigen-driven repertoires, CDR - complementarity-determining region, FWR - framework region

Coelho et al (16) previously showed that the presence of glycosylation moieties in immunoglobulin variable regions enabled B-cells to keep the impetus for B-cell activation and proliferation by interacting with lectin-expressing cells such as macrophages or dendritic cells (DC). Strikingly, the anti-PPS immune response is also engendered by a similar interaction with macrophages and DCs (17, 18) via a T-independent process. Hence, we included anti-PPS IgG sequences as positive controls for a lectin-binding based B cell selection.

When we compared IgG sequences with acquired N-glycosylation sites (IgG-Nglyc) from autoimmune diseases against anti-SpA IgG and anti-PPS IgG, our results indicated that both anti-SpA IgG and IgG-Nglycs from autoimmune diseases showed significantly greater negative selection within FWRs compared to anti-PPS IgG, indicating a strong conservation of these

domains in the former two groups (Figure 4). Interestingly, although PPS is a classical antigen, the selection pressures on anti-PPS IgG also showed negative selection at the CDRs, similar to potentially N-glycosylated IgG from autoimmune diseases and anti-SpA IgG. These results strongly suggested that the acquisition of N-glycosylated motifs in the CDRs and FWRs of IgG sequences from autoimmune diseases exhibit selection pressures homologous to certain B cell superantigens and T-independent antigens such as PPS.

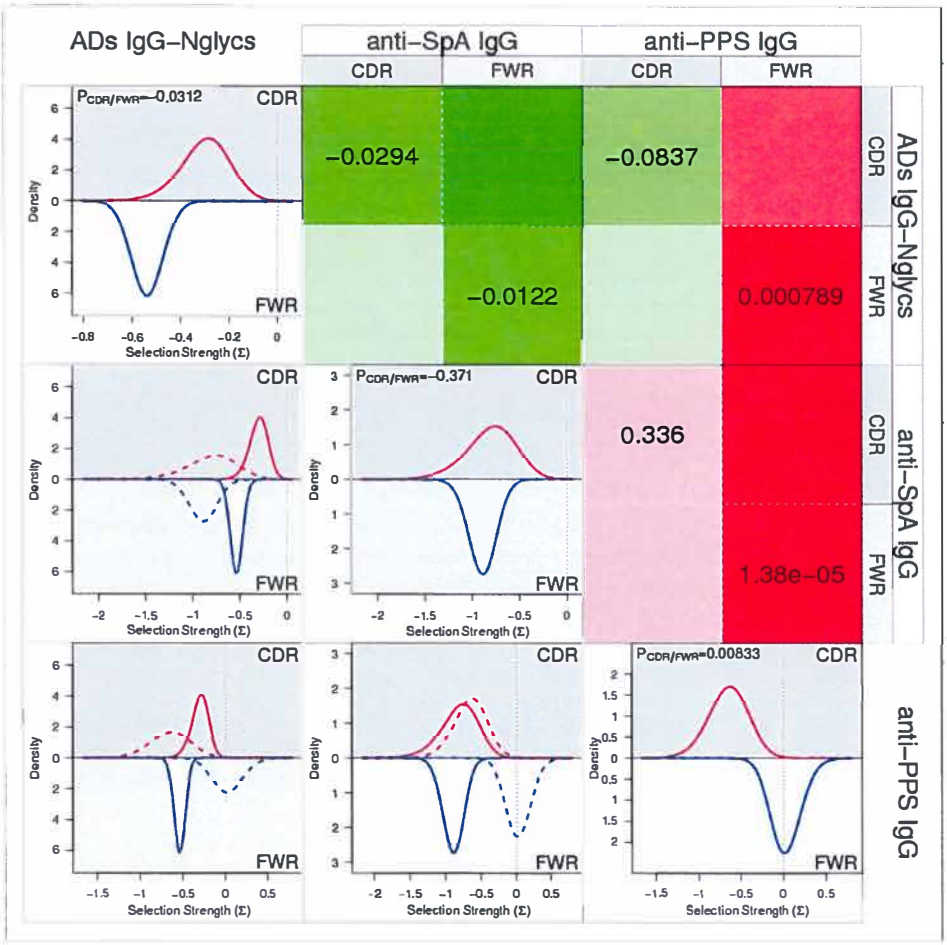

Figure 3: Selection pressures in IgG sequences with acquired N-glycosylation motifs. The selection pressures in IgG sequences with acquired N-glycosylation motifs from autoimmune diseases were compared to anti-SpA IgG and anti-PPS IgG in a two-point table format with the numbers indicating statistical difference between parameters in a particular column over that in the corresponding row. Negative sign and green colour indicate negative selection pressures, while red clour indicates positive selection . The strength of the selection pressure is also indicated with varying intensities of the colour codes. Abbreviations used are as follows: AD - autoimmune disease datasets, CDR - complementarity-determining region, FWR - framework region.

We did not include a comparison of IgG sequences with N-glycosylated motifs from control and antigen-driven repertoires because only 1/527 sequences had acquired N-glycosylation motifs in the control group, while in the classical antigen-driven repertoires, only 8/521 had such motifs. This is in contrast to the 50/375 motifs found in IgGs from autoimmune diseases.

DISCUSSION

This study clearly indicates that the B cell repertoire in autoimmune diseases are influenced by selection pressures different from those demonstrated in a classical (auto)antigen-driven response. We also observed that the autoimmune BCR repertoire was shaped by unconventional selection pressures that mimic B cell-superantigen interactions in its independency from antigen-driven BCR modulation. In our analysis, we clearly differentiated between different kinds of selection, where classical antigen-driven B cell selection is described as a response in which B cells that can moderately bind an antigen, go on to stimulate germinal center reactions where the process of SHM, differentiation and proliferation result in the production of clonal B cell populations with varying affinities to different epitopes of the antigen. On the other hand, superantigens interact with existing B cells, mostly at the conserved (FWR) regions of the BCR repertoire and are not necessarily influenced by BCR optimizations through SHM in conventional antigen binding regions (CDRs).

Our aim in this study was to use the relatively new analytical method known as BASELINe (15) to study how selection pressures correlated with the increased acquisition of N-glycosylation sites in BCR repertoires of autoimmune diseases compared to control and antigen-specific datasets. This strategy is different from the Focused-z test (3) that we used in our previous study (4), in that the Focused-z test only facilitated the detection of sequences showing statistically significant selection pressures, but did not offer a way to quantify the large number of sequences where selection pressures were not statistically significant and merely indicated trends. BASELINe on the other hand allows the cummulative quantification of selection pressures within a repertoire, as well as a tandem comparison of selection pressures between different experimental groups (15).

Results from using the BASELINe tool indicated that the IGHV repertoire in autoimmune diseases showed significant negative selection both in the CDRs and FWRs compared to controls and classical antigen-driven repertoires (Figure 1). This meant that there was almost no sign of antigen-driven selection in the conventional antigen binding sites or CDRs in the autoimmune repertoire, a stark contrast to classical antigen-driven repertoires. Meanwhile, in the FWRs, a strong emphasis on the conservation of immunoglobulin structure was noted. This pattern of selection was evident in the IgG-producing cell populations (Figure 2) as opposed to IgM and IgA isotypes (data not shown). This observation gained importance when we compared IgG sequences from autoimmune diseases against anti-SpA IgG. Greater negative selection pressures in the FWRs and CDRs of anti-SpA IgG is not surprising because SpA mostly targets highly conserved FWRs of IGHV3-expressing B cells and does not selectively provoke a modulation of the BCR repertoire based on the CDRs. However, in the autoimmune IgG repertoire, a strong emphasis on the conservation of FWRs and significant negative selection in CDRs, similar to that exhibited in anti-SpA IgG (Figure 2) suggests that there are similarities in the pattern of B cell selection in autoimmune repertoires and superantigen responses. Adding to this conclusion was the observation that the selection patterns of both autoimmune and anti-SpA IgG sequences were significantly different in every way from control and classical antigen-specific repertoires.

We then sought to understand how increased acquisition of N-glycosylation motifs in IgG sequences correlated with the unique selection pressures observed in autoimmune diseases. In our previous study regarding acquisition of N-glycosylation sites [Chapter 5], we had included anti-SpA sequences among antigen-selected datasets and observed that none of the 33 anti-SpA sequences analyzed acquired any N-glycosylation sites. This fits our hypothesis that acquisition of N-glycosylation motifs may be an alternative form of B cell selection that mimics BCR-superantigen interactions at least within the FWRs. Instead of superantigen binding and stimulation, the sugar moieties at the acquired N-glycosylation sites may interact with lectins of the innate immune system and stimulate a response.

However, we were still unsure if these acquired N-glycosylation motifs could be a result of their role in enhancing antigen-specific binding (19, 20) or whether they contributed to a non-antigen-driven stimulation. Since both B cells with glycosylated immunoglobulins and PPS antigens activate the immune system by binding to lectin-expressing cells such as macrophages and dendritic cells (16-18) we included anti-PPS IgG sequences as positive controls for a T-independent and lectin-binding based B cell selection.

It is to be noted that in our previous study, we had included 100 anti-PPS IgG sequences within antigen-driven datasets. As in the case of anti-SpA sequences, none of the anti-PPS IgG sequences carried acquired N-glycosylation motifs. This is understandable in light of the fact that PPS is also a polysaccharide or sugar entity similar to that on N-glycosylated BCRs. We hypothesized that the similarities in composition and ability to interact with lectins of the innate immune system might also translate as similarity in selection pressures between anti-PPS immunoglobulins and glycosylated BCRs in autoimmune repertoires. This theory was considerably supported by the observation that IgGs with acquired N-glycosylation motifs from autoimmune diseases and anti-PPS IgG showed similar patterns of negative selection in their CDRs (Figure 4). However, unlike IgGs from autoimmune diseases and anti-SpA IgG, anti-PPS IgGs showed no selection in their FWRs.

All of the above observations suggested that the common selection pressures shared by IgG-producing B cells in autoimmune diseases and superantigen responses may lay the background on which the acquisition of N-glycosylation motifs on BCR variable regions could add a second selective stimulus through binding to lectins of the innate immune system.

It is possible that not all autoimmune diseases will show the same pattern of selection pressures as seen in this study. This may be due to basic differences in selection pressures acting on these repertoires compared to the autoimmune diseases studied here. It would be interesting to evaluate the type and extent of selection pressures in autoimmune diseases where B cell selection may be truly (auto)antigen driven and compare them with the diseases analyzed in this study.

The association of IgG with unique selection pressures such as acquisition of N-glycosylation motifs (4, 13), conservation of FWRs and lack of positive selection in CDRs strongly suggest an isotype-specific functional outcome for these differentially-selected IgG-producing cells, which may mediate the inflammatory pathology seen in autoimmune diseases through different effector mechanisms. All the above observations provided clear support for our hypothesis that selection pressures influencing B cell selection in autoimmune diseases are very different from those in classical antigen-driven repertoires and are more similar to superantigen-based and T-independent selection.

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7 GENERAL DISCUSSION: A HYPOTHETICAL MODEL FOR ALTERED B CELL SELECTION IN AUTOIMMUNE DISEASES

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Manuscript in preparation as Review

INTRODUCTION

Autoimmune diseases are characterized as conditions where the immune system apparently loses the ability to distinguish between self and non-self. Although, more than 80 different autoimmune diseases are known to exist, the etiology behind these conditions are yet to be elucidated (1). They are generally classified into two types: systemic and organ-specific autoimmune diseases. Systemic autoimmune diseases are those that affect multiple organs and are usually characterized by the production of autoantibodies that recognize a diverse array of cytoplasmic and nuclear antigens. Organ-specific autoimmune diseases, such as Sjögren's syndrome, on the other hand, often display autoantibodies that bind disease-specific (auto)antigens within particular glands or organs. There is growing evidence that genetics, environmental exposure and/or hormonal imbalances may play a role in conferring susceptibility to autoimmune diseases.

Of all possible agents, the role of environmental exposures to infections are thought to be major players in the development of autoimmune diseases, especially given the occurrence of disease onset after certain infections, seasonal autoimmune diseases flares (similar to seasonal infections) and the isolation of microbial DNA or RNA from diseased tissues of patients with autoimmune diseases. Examples are: periodontal disease is associated with rheumatoid arthritis (RA) (2), Staphylococcal infections are associated with granulomatosis with polyangiitis (formerly known as Wegener's Granulomatosis) (3), autoimmune thyroid diseases are postulated to develop as a result of a synergistic interplay between hepatitis C virus infection and interferon-α (IFNα) therapy (4) and human cytomegalovirus (HCMV) is epidemiologically correlated with a number of autoimmune diseases such as RA (5). The association between infection and autoimmunity is also demonstrated in animal models such as Theiler's murine encephalomyelitis virus-induced demyelinating disease (TMEV-IDD) which is a model for human multiple sclerosis (5); herpes simplex virus (HSV)-associated stromal keratitis in which HSV infection leads specific T-cell responses against corneal antigens in both humans and mice (7, 8) and autoimmune myocarditis associated with coxsackie virus infection (9).

Even non-infectious agents such as adjuvants present within vaccines are associated with the incidence of autoimmune diseases and the phenomenon is referred to as 'Autoimmune Syndrome Induced by Adjuvants (ASIA) (10). Pathogens and adjuvants may initiate or perpetuate autoimmunity through several mechanisms such as molecular mimicry, bystander activation, deficiency in clearance of apoptotic/necrotic cells after infections and as superantigens (5).

Evidence is particularly building up in the case of superantigens, a certain class of antigens, and their possible role in the pathogenesis of autoimmune diseases. According to studies on T-cell-activating factors, while conventional peptide antigens interact with less than 0.1% of the naive lymphocyte repertoire, a superantigen promotes supraclonal responses within a large proportion (often more than 5%) of the lymphocyte pool (11)

Superantigens were originally thought to act primarily via their simultaneous binding to human major histocompatibility complex (MHC) class II molecules (primarily HLA-DR) and T-cell antigen receptors (TCRs) irrespective of the antigen-specificity of the TCR (12, 13). Later studies then proved that superantigens could also bind solely to the complementarity-determining regions (CDR) and framework regions (FWR) of the variable domain (V) of the β subunit of T cell receptors (TCR) and act as wedges between the TCR and MHC to divert an antigen-MHC complex away from the TCR combining site (13). In this way, superantigens are able to displace the normal mechanism for T-cell activation by specific antigen-MHC complexes. (14). Moreover, different superantigens exhibit highest affinities for different Vβ subsets of TCRs (15).

Superantigens also show varying binding specificities to different HLA-DR allotypes (16). Interestingly, susceptibility to a number of autoimmune diseases, including rheumatoid arthritis (17), multiple sclerosis (18) and autoimmune thyroiditis (19), is also associated with particular alleles of HLA-DR genes. This effectively bolsters the premise that autoimmune diseases could potentially result from the two-hits of genetics and environmental exposure to (superanigenic) infections.

Further studies showed that superantigen interactions are not restricted to T cells and MHCs, but that it extends to B cells as well. As in the case of T cell superantigens, B cell interactions with superantigens also result in activation, proliferation, deletion or migration of these cells (10). Given this wide range of immunomodulating abilities, it was inevitable that superantigens would be linked to the etiology of autoimmunity (5, 20). However, concrete evidence for this link has been elusive. While there are several studies correlating the presence of T cell superantigens with the incidence of autoimmune diseases in humans and mice, such studies are fewer in the case of B cell superantigens in humans. Although studies were carried out in experimental mice models, they offer little towards understanding the precise role of superantigens in B cell-mediated autoimmune disorders in humans.

In this chapter, we offer a unique and novel hypothesis for altered B cell selection that possibly links B cell superantigens with autoimmune diseases. Our hypothesis is largely based on evidences from the study of immunoglobulin gene rearrangements in autoimmune diseases and we will discuss this in the context of our research and existing paradigms. Given the discussion over enlisting superantigens as potential targeted therapies for autoimmune diseases (4, 21), the observations made in this review may have significant implications for the research and development of such therapies.

SUPERANTIGENS AND AUTOIMMUNITY

The main idea behind the hypothesis associating autoimmunity with superantigens is that they do not just aggravate autoimmune diseases but may also be central in converting tolerable, controllable immune responses into relentless, uncontrollable disasters for the host (19). Indirect experimental correlations and epidemiological studies are suggestive of the involvement of superantigens in autoimmune disorders. High levels of circulating Vβ2 T cells in patients with acute Kawasaki disease were significantly correlated to the incidence of superantigen-expressing Staphylococcus aureus or group A streptococcus (GAS). These superantigenic infections are known to stimulate Vβ2+ T cells (22). Another study which reinforced the concept of the superantigen-autoimmunity link was the observation that an endogenous retroviral-derived superantigen was actually a candidate gene for autoimmune type 1 diabetes (23). In this report, it was observed that V-restricted T cell proliferation was mediated by monocytes transfected with sequences that encode the retroviral-derived superantigen. In all the above scenarios, the transition from infection to autoimmunity was attributed to superantigen influence on T cells. We will now proceed to dissect possible mechanisms underlying a potential transition from infection to autoimmunity in the context of B cell superantigens and autoimmune diseases.

B cell superantigens

The most well-characterized B cell superantigens are the Staphylococcal protein A (SpA), the Peptostreptococcus magnus protein L (PpL), the endogenous human gut-associated sialoprotein pFv and the HIV-1 envelope protein gp120. The SpA, pFv and gp120 superantigens interact with

evolutionarily conserved IGHV framework regions (FWRs) of the B cell receptor (BCR) and can stimulate B cell differentiation, proliferation and Ig secretion (10, 24). SpA interacts specifically between residues 75 and 84 of the FWR3 region within IGHV3 sequences (25), which is the largest IGHV gene family in normal individuals and this dominance is unaffected in many autoimmune diseases (26-29). The gp120 and pFv proteins also share a similar affinity to the same or nearby FWR3 sites, again on IGHV3 genes, as was shown by competitive binding experiments with SpA (10, 24). Not only does binding of superantigens to these FWR3 sites result in B cell activation independent of conventional antigen binding sites in the CDRs, but it also ensures the activation of 30-50% of the B cells in humans. Added to this, SpA also has the ability to bind to the heavy chain constant region of IgG immunoglobulins (10) possibly resulting in polyclonal stimulation.

PpL, on the other hand, has domains which bind specifically to conserved FWRs of the immunoglobulin light chain genes VL1, VL3 and VL4. This ensures the interaction of PpL with more than half of all circulating B cells in humans (30), which again, is an essential characteristic of a superantigen (10).

LINKING B CELL SUPERANTIGENS AND AUTOIMMUNITY

Common emphasis on conservation of Ig framework regions and lack of positive selection on complementarity-determining regions

BCR-superantigen interactions in the examples mentioned above indicate that conservation of heavy and light chain FWR region structure within the BCR is instrumental for B cell superantigen-mediated effects. Interestingly, in chapter 6 of this thesis, we were able to confirm such a conservation of FWRs in SpA reactive-B cells expressing the IgG isotype using experimentally validated computational analyses, known as Bayesian estimation of Antigen-driven SELectIoN or BASELINe (31). We also observed (in chapter 4) a similar emphasis on maintenance of immunoglobulin FWR structural integrity within IgG sequences from diseased parotid salivary glands of patients with primary Sjögren's syndrome (pSS), a classic example of an autoimmune disease, in comparison to non-pSS individuals (32). In chapter 6, we extrapolated this analysis to include IgG sequences from different autoimmune disorders and compared them against IgG sequences extracted from control tissues of healthy and non-autoimmune individuals. Again, we observed that the FWRs of IgG sequences from autoimmune diseases were more conserved than those in normal controls.

A surprising and crucial finding was that IgG sequences from autoimmune disorders exhibited almost no sign of positive selection pressures in their CDRs (31). Moreover, the IgG sequences from classical antigen-driven repertoires were more positively selected in both their CDRs and FWRs than the IgG sequences from autoimmune diseases.

These observations were initially puzzling in the context of the hyperactivation and proliferation of B cells that is generally associated with autoimmune disorders and is assumed to be antigen-driven (33, 34). However, these previous reports (33, 34) did not differentiate between mutations introduced during affinity maturation and mutations that occur as a result of repeated cell divisions (as seen in non-Ig genes). They also did not take into account the bias for mutational hotspots and the possible differences in selection pressures between CDRs and FWRs. All of these factors are considered within the Focused-z test (also known as Focused Binomial test) (35) and BASELINe software (36) used in our studies (chapters 4 and 6, respectively). Hence, the observation of almost no positive antigen selection in IgG sequences from autoimmune diseases

raises the possibility that B cells in autoimmune repertoires may be generated by unconventional selection pressures that may not necessarily involve classical protein (auto)antigens.

Increased prevalence of N-glycosylation motifs in Fab regions of IgG from autoimmune diseases

Given the minimal evidence for positive antigen selection pressure in the IGHV sequences of B cells from pSS patients and other autoimmune diseases, we speculated that these B cells may be selected based on certain properties of the immunoglobulins produced rather than on the specificity of antibody-antigen interactions. Since a previous simulation study of germinal center reaction models associated the acquisition of N-glycosylation motifs with conferring a selective advantage to B cells (37), we evaluated if acquired N-glycosylation patterns could be one of these properties.

During the modulation of the Ig repertoire, the somatic hypermutation (SHM) process may introduce mutations in the variable regions of both heavy and light chains that either create new (acquired) N-glycosylation sites or destroy such sites present in a germline variable gene. Studies on immunoglobulin variable regions revealed higher frequencies of acquired N-glycosylation sites in follicular lymphomas (FL) compared to other B cell malignancies and normal somatically mutated memory B cells (38, 39). The relative lack of N-glycosylation motifs in the immunoglobulin variable (V) regions of normal memory B cells, normal IgM and IgA plasma cells and non-FL B-cell lymphomas suggested that the frequency of N-glycosylation sites in immunoglobulin V-regions is generally quite low in post-germinal center Ig-producing cells (38, 40).

In contrast to this, we observed (chapter 4) that a high proportion (~25%) of the IgG sequences from diseased parotid glands of pSS patients exhibited acquired N-glycosylation sites in their IGHV regions (32). This suggested that a proportion of IgG-expressing cells within the parotid glands of pSS patients were probably selected on the basis of their glycosylation patterns. The acquired N-glycosylation sites occurred both within CDRs and FWRs. Since CDRs are conventional antigen-binding sites, the increased tendency for acquisition of sugar moieties within these regions in autoimmune diseases could likely indicate some sort of B cell selection. However, with regards to acquired N-glycosylation sites in FWRs, a striking observation was that nearly 60% of these sites were noted in the FWR3 domain of IGHV3 genes and were created by mutations at residues 75 to 84. Remarkably, these residues are precisely the same sites and region of FWR3 which is associated with B cell-SAg interactions by SpA, gp120 and pFv proteins, as discussed above (10).

Interestingly, all the above phenomena were also observed to be true for IgG sequences from other autoimmune disorders such as SLE, RA, Chaga's disease and multiple sclerosis (41). In addition to acquired N-glycosylation motifs within CDRs, 53% of the total acquired N-glycosylation sites in IgG sequences from autoimmune diseases expressing IGHV3 genes occurred within residues 75-84 of FWR3. As a result of all these observations, we speculate that if B cell-SAg interactions within the FWRs can result in B cell proliferation, activation and Ig secretion (10), it is highly conceivable that the acquisition of sugar moieties within FWRs may also provide analogous triggers for B cell hyperactivation through interactions with sugar-binding proteins such as lectins.

Correlation between acquisition of N-glycosylation motifs and selection pressures in autoimmune diseases

We now sought to understand how increased acquisition of N-glycosylation motifs (chapter 5) correlated with the selection pressures observed in the IGHV repertoires of autoimmune

diseases. Coelho et al (42) previously showed that the presence of glycosylation moieties in immunoglobulin variable regions promoted malignant B cell activation and proliferation by interacting with C-type lectins (such as mannose receptors and DC-SIGNs) on macrophages or dendritic cells (DC). Strikingly, the immune response against the pneumococcal polysaccharide antigen PPS, is also engendered through the interaction of PPS antigens with lectins on macrophages and DCs (43, 44) via a T-independent process. This is somewhat analogous to the interaction of glycosylated moieties on BCRs with macrophage and DC lectins described by Coelho et al. Hence, in our analysis of B cell selection pressures in autoimmune diseases (31), we also included anti-PPS IgG sequences as positive controls for a lectin-binding based B cell activation and selection (chapter 6).

We compared IgG sequences with acquired N-glycosylation sites (IgG-Nglyc) from autoimmune diseases with SpA-reactive IgG and anti-PPS IgG sequences. Our results indicated that the selection pressures on anti-PPS IgG sequences showed negative selection at the CDRs, similar to IgG-Nglycs from autoimmune diseases. This makes the anti-PPS B cell response significantly different from a classical protein antigen-driven response (31) because the latter response is characterized by positive selection in the CDRs (Chapter 6). Moreover, both SpA-reactive IgG and IgG-Nglycs from autoimmune diseases showed significantly greater negative selection within FWRs compared to anti-PPS IgG, indicating a strong conservation of these domains in the former two groups. This indicates that the B cells with acquired N-glycosylation motifs from autoimmune diseases exhibits selection pressures similar to B cells against polysaccharide (PPS) antigens in their conventional antigen-binding sites (CDRs), possibly due to their common interaction with lectins for mediating selection and survival. Moreover, the overall pattern of selective pressures (in CDRs and FWRs) exhibited by IgG-Nglycs in autoimmune diseases sets them clearly apart from classical antigen-driven responses (31).

Therefore, our analysis of acquired N-glycosyation motifs in IgG sequences from autoimmune diseases suggests that the presence of such motifs in both FWRs and CDRs could contribute to the selection and survival of B cells (as seen in SpA-reactive B cells and anti-PPS B cells, respectively).

B cell selection in autoimmune diseases resemble superantigen-derived B cell response

In chapter 6, we established that the IgG sequences from autoimmune diseases were significantly different to IgG sequences from normal/non-autoimmune repertoires and classical antigen-driven repertoires and more similar to superantigen-derived B cell response. Then, we compared the IgG sequences from autoimmune diseases (AD IgG) with SpA-reactive IgG. We observed that only the CDRs of IgG sequences from autoimmune diseases were significantly less negatively selected (less conserved) in AD-IgG than SpA-reactive IgG, while no difference in selection was noted in the case of FWRs (Figure 2).

We further compared SpA-reactive IgG and IgG sequences with acquired N-glycosylation motifs (ADs IgG-Nglycs) (Figure 3). This analysis showed that both CDRs and FWRs were significantly less conserved in AD IgG-Nglycs compared to SpA-reactive IgG. These observations suggested the presence of an initial altered selection pressure on IgG-producing B cells in autoimmune diseases which made it different to normal/non-autoimmune repertoires. This altered selection appeared to lay the background on which the acquisition of N-glycosylation motifs due to SHM on BCR variable regions could add a second selective signal or activation through binding to lectins of the innate immune system.

However, the question still remained as to what factors could induce the initial altering of B cell selection pressures in autoimmune diseases to resemble those of superantigen-derived B cell responses and differ from normal controls. In this regard, we speculated that our studies may tentatively provide a first evidence for the much debated and to date unproven link between B cell-superantigen infections and autoimmunity.

A novel model for B cell selection in autoimmune diseases

Generally, B cell hyperactivation and autoantibody production in autoimmune diseases are believed to occur through classical autoantigen-driven selection involving antigen presentation by MHC molecules to T cells. Alternatively autoantigen-driven B cell activation could also be augmented through Toll-like receptor (TLR)-mediated activation by auto-antigens such as RNA-complexes (TLR7) or DNA-complexes (TLR9) (45-48). In these pathways (Figure 1, I and II), B cells are proposed to be activated through interactions with autoantigens that are present in excess within diseased tissues due to defective clearance after apoptosis/necrosis, autoantigens that may have been genetically altered in some way or due to autoantigens that share some structural similarity with antigens from pathogenic organisms (molecular mimicry). In the model for classical autoantigen-driven autoimmunity, BCR-mediated signal transduction depends on receptor density as well as antigen affinity and antigen dose (49). On the other hand, in the TLR-mediated pathway model, B cell activation in autoimmune diseases is prompted through ligation of BCR and specific TLRs by endogenous ligands, resulting in enhanced autoantigen presentation by these B cells, cytokine secretion, the facilitation of autoreactive T-cell engagement and autoantibody secretion (50). TLR-mediated B cell activation is supported by the existence of certain B cells such as age-associated B cells that respond poorly to BCR and CD40 ligation but are stimulated by ligation of TLRs such as TLR9 and TLR7 in autoimmune experimental models and human autoimmune diseases (51, 52).

Our studies lead us to propose a third alternative or additional form of B cell selection in autoimmune diseases which is depicted in Figure 1, III. In this model, which we refer to as the 'altered B cell selection model', we propose that infections by certain pathogens producing B cell-superantigens may contribute to an initial skewed B cell repertoire. We termed it altered selection because unlike classical antigen-driven selection, superantigen-derived B cell selection occurs independent of conventional antigen-binding sites and the process of antigen affinity maturation.

We hypothesized that in individuals with susceptibility factors (eg. hormonal imbalances or genetics) predisposing towards autoimmunity, a proportion of the B cells selected for survival following superantigenic infections may be further selected or activated due to their possessing certain unique characteristics such as N-glycosylation moieties on their Fab region. In fact, this possibility is strongly indicated in the case of the female hormone, progesterone, which can modulate N-glycosylation at the Fab region through its influence on oligosaccharyltransferase expression (53). Given the fact that certain autoimmune diseases have a high preponderance of female versus male incidence (54), it is possible that an initial altered B cell selection combined with hormonal imbalances or genetic factors may provide the predisposing background for continued B cell differentiation and proliferation, ultimately leading to autoimmune disease.

B cells with glycosylated BCRs, may help to perpetuate the autoimmune response by binding with lectins of the innate immune system, such as C-type lectins on dendritic cells (42). Interactions with lectins may drive B-cell proliferation and ongoing SHM within diseased tissues of autoimmune patients in a non-classical way, independent of antigen-binding specificity and

accelerated by cytokines such as BAFF (B cell activating factor). Such interactions may also occur outside of germinal centers (GCs), as suggested by William et al. who showed evidence for proliferation, SHM and (auto)antibody formation outside classical GCs in murine spleen (55). Furthermore, the significant prevalence of IgG-producing cells with altered selection pressures and acquired N-glycosylation sites indicate that the selection of these B cells is associated with class-switching and specific effector functions.

Autoantibodies explained using the altered B cell selection model in autoimmune diseases

In our studies (chapters 4-6), we had no knowledge of the antigen-specificities of the sequences analyzed within the autoimmune repertoires (31, 41, 32). Hence, we cannot make any conclusions regarding the presence of characteristic autoantibodies in certain

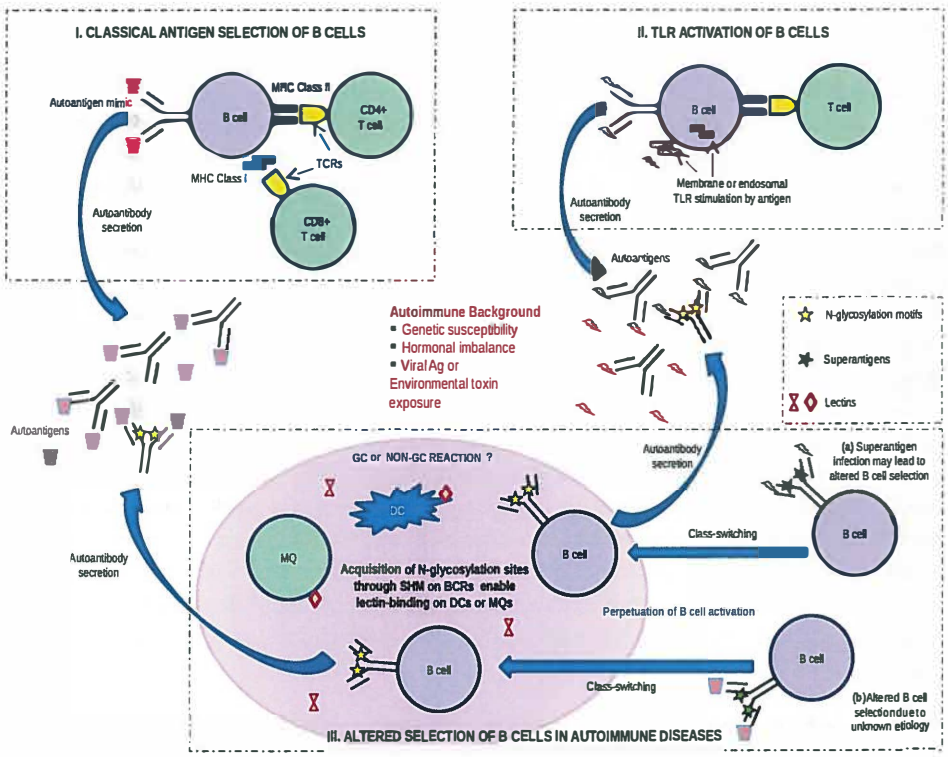

Figure 1: Hypothetical model for the role of altered B cell selection in autoimmune diseases.B cell selection and hyperactivation in autoimmune diseases are conventionally believed to occur through classical (auto) antigen-driven selection (I) and/or TLR-mediated activation (II). However, our studies on B cell gene repertoires in autoimmune diseases lead us to propose a third alternative pathway for B cell selection and activation in autoimmune diseases. In this model, infections by pathogens producing B cell-superantigens may contribute to an initial altered selection of the B cell repertoire. In individuals with a background of autoimmune susceptibility, Once selected (and activated), these B cells (including autoreactive B cells) probably undergo SHM that allows them to acquire N-glycosylation motifs and further suvive through lectin interactions. [Abbreviations: DC - dendritic cells, MQ – macrophages, GC-germinal center]

autoimmune diseases, such as anti-Ro/SSA and anti-La/SSB autoantibodies in pSS and anti-DNA autoantibodies in SLE. However, our model for altered B cell selection in autoimmune diseases does offer some avenue for speculation in this regard.

Previous studies have shown that many autoimmune disease-associated autoantibodies are also present naturally in healthy individuals but tend to be quantitatively increased and more (auto) antigen-specific in diseased individuals (56). Moreover, these natural autoantibodies are germline-encoded (57), which suggests that in all probability, they will exhibit conservation of their FWRs and CDRs. Hence, superantigen exposure could, at least theoretically, lead to an initial selection of natural autoantibody-producing B cells (alongwith other B cells) due to their binding to the conserved IGHV regions of the BCRs.

Our studies showed that although the B cell repertoires (specifically IgG) from autoimmune diseases were highly mutated, they exhibited conservation of FWRs and less/no negative selection within their CDRs. But both of these selective pressures were significantly less than superantigen-selected B cells (Chapter 6) (32). Hence, according to our model of altered B cell selection, we hypothesize that superantigenic infections (such as SpA) could create an initial selection of unmutated/less mutated B cells (including natural autoantibody-producing B cells) in autoimmunity-prone individuals. Once selected, these B cells (including autoreactive B cells) probably undergo SHM that allows them to acquire N-glycosylation motifs and further survive through lectin interactions. In addition to this, the natural autoantibody-producing B cells could also acquire fine-epitope specificity for autoantigens due to SHM (58). This increased affinity for autoantigens and enhanced survival (through acquisition of N-glycosylation motifs) may even set the stage for breaking tolerance. Thus, our model for altered B cell selection suggests that the selective pressures which shape the B cell repertoires in autoimmune diseases could also inadvertently select natural autoantibody-producing B cells. A first step towards confirming this hypothesis would be to analyze the selective pressures exhibited by Ig sequences from various autoantigen-specific B cells from different autoimmune diseases and will be the focus of future research by our group.

As proposed by Polly Matzinger in her Danger Model for explaining the basis of autoimmunity, the antigen-specificity of autoantibodies generated in different autoimmune diseases may be dictated by the tissue or gland affected (59). However, according to our proposed model, we speculate that the type of autoantibody-producing B cells selected in autoimmune diseases may result from specific superantigenic infections/exposures at certain sites or tissues in genetically predisposed individuals. This is indicated by the facts that autoimmunity is organized around a particular set of auto-antigens (58) and that patients with autoimmune diseases were observed to have relatively higher titers of natural autoantibodies prior to developing disease symptoms, than unaffected individuals (60).

Effect of B cell depletion on B cell selection pressures in autoimmune diseases

Our group was the first to study changes in B cell repertoires after B cell depletion therapy in autoimmune diseases. This analysis (chapter 4) was carried out using the Focused test software, on the IGHV3 repertoire in diseased parotid salivary glands of five pSS patients treated with rituximab (RTX) (32). However, only sequences showing significant selection pressures were included in that analysis. In this chapter, we analyzed the entire IGHV3 repertoire from the same 5 pSS patients analyzed in chapter 4, by using the BASELINe software.

We observed a strong emphasis on conservation of FWR structure and no signs of positive selection within CDRs in IgG sequences from pSS patients at baseline (Figure 2). Surprisingly, despite the B cell depletion effect of RTX, which also extended to the parotid glands to a moderate degree (61, 62), there was no significant change in the pattern of B cell repertoires at 16 weeks and at 36-52 weeks after RTX compared to baseline. This lack of change was also seen in the prevalence of acquired N-glycosylation motifs in the IgG sequences from our pSS patients (chapter 4), where no significant alteration was seen at 16 weeks and at 36-52 weeks after RTX (32). This suggests that RTX therapy in pSS patients does not result in any alteration to certain core characteristics of the B cell repertoire in pSS and this persistent B cell repertoire could possibly contribute to the recurrence of pSS symptoms observed in patients after RTX treatment.

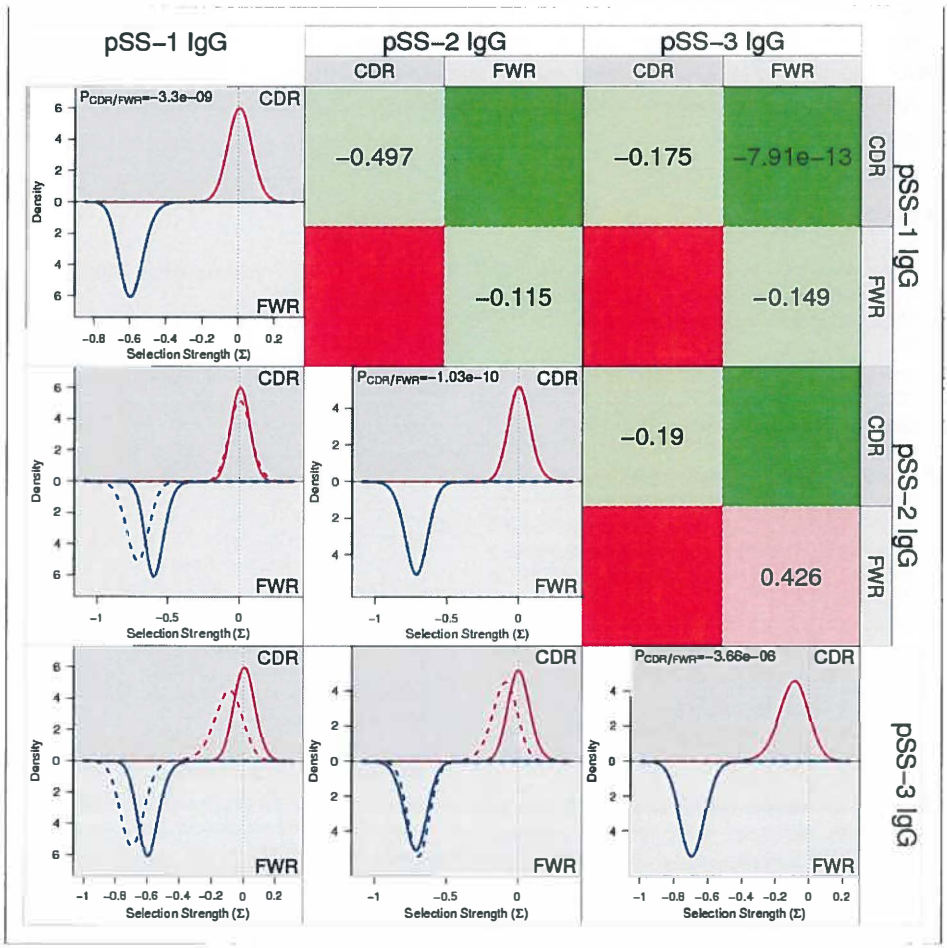

Figure 2: Selection pressures on IgG-producing cells in pSS patients before and after RTX. Selection pressures exhibited by IGHV regions of IgG sequences from diseased parotid salivary glands of pSS patients were compared at baseline (pSS-1), 16 weeks after RTX (pSS-2) and at 36-52 weeks after RTX using the software called BASELINe. No significant changes in selection pressures were observed within the IGHV regions due to RTX treatment.

CONCLUSIONS AND IMPLICATIONS OF THIS THESIS

Through our studies, we provide evidence for the different selective pressures exhibited by B cell repertoires in autoimmune diseases. We also prove that the forces influencing the generation of the autoimmune repertoire is significantly different from classical antigen-driven B cell repertoires and is more reminiscent of superantigen-derived B cell repertoires. In addition to this, our studies (Chapters 4-6) suggest that the acquisition of N-glycosylation motifs in IGHV genes of the autoimmune repertoire could indicate a SHM-based selection pressure on B cells. More importantly, using the above evidences from computational immunology, we also provide a coherent model for a link between superantigens and autoimmune diseases. To our knowledge, our group is the first to state all these conclusions and our findings may spur a new direction for further research into the role of B cells in autoimmune diseases.

We speculate that our findings could also provide a strong rationale for the research and development of alternative therapies that seek to modulate autoimmune diseases through the use of anti-glycosylating compounds (64) or glycans that can competitively bind to lectins on dendritic cells and thereby interfere with the recruitment of a deleterious (auto)immune response (65).

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THESIS SUMMARY

This thesis focused on the study of immunoglobulin variable heavy chain (IGHV) genes in autoimmune diseases with an emphasis on primary Sjögren's syndrome (pSS) in the first few chapters. From our studies on pSS patients, we were able to uncover certain novel insights. We then extrapolated the same strategy to study other autoimmune diseases. Our studies on IGHV gene sequences encoding for a part of the B cell receptor (BCR) revealed novel and significant features regarding the selection of B cells in autoimmune diseases.

The relevance of B cells to autoimmune diseases such as pSS, rheumatoid arthritis (RA) and systemic lupus erythrematosus (SLE) became clear when B cell depletion therapies proved to be successful in providing palpable clinical relief in treated patients (1). In addition to the therapeutic efficacy, analyzing the repopulation of B cells after B cell depletion also provides an idea of the B cell populations that may be implicated in disease symptoms and recurrence after therapy over time. This rationale formed the basis of our initial study (Chapter 3) on pSS patients treated with rituximab (RTX) (2), a B cell-targeting therapeutic antibody. In this study, we used IGHV sequences as a form of B cell marker to trace the presence of B cell clonal populations before and after treatment. A significant aspect of this study was that we had access to parotid biopsies not just from baseline and 12-16 weeks after RTX, but also from 36-52 weeks after RTX, a time point when the signs of disease relapse become evident. As a result, we were able to detect certain immunoglobulin-producing cells before RTX treatment whose clones persisted in salivary glands of pSS patients even after B-cell depletion with RTX. The presence of mixed isotype expression within these groups of clonally-related cells indicated the presence of localized class-switching within salivary glands of pSS patients. From this study, we speculated that persistent immunoglobulin-producing cells could underlie disease relapse after treatment (2).

Our next step was to evaluate the changes taking place within the immunoglobulin repertoire in diseased parotid glands of pSS patients as a result of RTX treatment. This formed the basis of our study in Chapter 4. At first, we determined the features that were characteristic of the immunoglobulin-producing cells within diseased parotid glands from pSS patients by compared them to immunoglobulin-producing cells obtained from control parotid biopsies of non-pSS patients. As a result, we observed the following features to be significantly increased in pSS patients at baseline: (a) clonal expansions of immunoglobulin-producing cells expressing IGHV-3 (b) expression of IgG1 versus other IgG subclasses (c) conservation of IgG framework regions (d) prevalence of acquired N-glycosylation sites in the variable heavy-chain regions of IgG sequences. B-cell depletion with RTX failed to reset these core characteristics of Ig-producing cell populations in treated pSS patients. These observations added evidence in favor of our previous conclusion that immunoglobulin-producing cells that persisted after RTX could contribute to the disease relapse observed after RTX (2). We also surmised that the transient clinical relief from SS symptoms after RTX was probably due to the ablation of B-cell and $CD20^+$ plasma cell numbers (3) resulting in lower levels of certain autoantibodies from short-lived plasma cells and/or reduction in other effector B-cell functions, such as antigen presentation and cytokine production (4, 5). We proposed that the underlying autoimmune mechanisms were probably maintained by long-lived plasma cells as has been indicated in studies in SLE (4, 6-8).

To our knowledge, this was also the first study to suggest a role for N-glycosylation in the selection of immunoglobulin-producing cells in pSS patients. We postulated that a proportion of B-cells in pSS patients could be selected on the basis of acquired N-glycosylation motifs in their Ig variable regions.

This lead us to wonder if such a phenomenon could also be true for other autoimmune diseases and resulted in our study described in Chapter 5. Our study clearly indicated a significant

selection of B cells with potentially N-glycosylated motifs that were acquired due to somatic hypermutation (SHM) in autoimmune diseases compared to normal or non-autoimmune (control) tissues and antigen-specific repertoires. This selection was even more evident within IgG-producing cell populations. We proposed a novel hypothesis that the presence of acquired N-glycosylation sites or motifs (ac-Nglycs) within IGHV sequences from certain autoimmune diseases could indicate an unconventional selection pressure that possibly enabled B-cells with glycosylated BCRs to engage, survive and persist within autoimmune lesions through their interaction with lectin-expressing cells such as dendritic cells or macrophages (9).

The most striking aspect of our results in Chapter 5 was the observation that a large proportion of ac-Nglycs consistently coincided with framework regions targeted by B-cell superantigens such as Staphylococcal protein A (SpA), endogenous human gut-associated sialoprotein, pFv and HIV-1 envelope protein, gp120. BCR-superantigen interactions characteristically occur at framework regions (FWRs) (10). For a B cell-superantigen, this ability ensures the availability of binding targets minimally influenced by the unpredictability of genetic recombination or the hypervariability introduced by SHM (mostly focused on complementarity-determining regions or CDRs). Thus, BCR-superantigen interactions indicated two important points; one, since interactions at these sites by superantigens lead to B cell activation, proliferation, differentiation and immunoglobulin secretion (10), a similar engagement of the BCR by other moieties could conceivably result in the same response. The second point was that the emphasis on maintaining overall structure of FWRs outside of classical antigen-binding sites (CDRs) could also be a sign of B cell selection. We proceeded to explore if such a form of B cell selection could be detected in autoimmune repertoires.

We had already observed in Chapter 4 that the IGHV sequences from diseased salivary glands of patients with pSS demonstrated significantly increased conservation of FWR structure compared to non-pSS control patients despite being heavily mutated [Hamza et al, submitted] which was reminiscent of BCR-superantigen interactions. Through our study in Chapter 6, we were able to confirm that emphasis on maintenance of FWR structure was also a characteristic of the IGHV repertoire in other autoimmune diseases such as RA, SLE, multiple sclerosis, Chaga's disease, granulomatosis with polyangiitis (formerly known as Wegener's granulomatosus) and ankylosing spondylitis. Moreover, the IGHV repertoire in autoimmune diseases showed a significant lack of positive selection in their CDRs, which are conventional antigen-binding sites. In these aspects, the autoimmune BCR repertoire was significantly different from both control and classical antigen-selected repertoires, while being very similar to superantigen-selected repertoires. Our observations suggested that the altered selection pressures of B cells in autoimmune diseases could lay the background on which the acquisition of N-glycosylation motifs on BCR variable regions could add a second selective signal or activation through binding to lectin-expressing cells of the innate immune system (9). This, in essence validated our hypothesis that altered B cell selection pressures in autoimmune diseases could underlie the autoimmune pathology.

As a result of our observations from Chapters 5 and 6, we used our evidences pertaining to altered B cell selection pressures in autoimmune diseases to present a hypothetical model for a possible link between superantigens and autoimmune pathology. In this model, we proposed that certain B cell-superantigen infections could contribute to an initial altered selection of the B cell repertoire. In individuals with susceptibility factors predisposing towards autoimmunity, superantigen infections could lead to the selection and activation of B cells with certain unique characteristics such as N-glycosylation of their Fab region. In fact, this mechanism was already

proven in the case of the female hormone, progesterone, which has the ability to modulate N-glycosylation at the Fab region through its influence on oligosaccharyltransferase expression (11). Given the fact that autoimmune diseases have a high preponderance of female versus male incidence (12), it is possible that an initial B cell hyperactivation combined with hormonal imbalances or genetic factors may provide the predisposing background for continued B cell differentiation and proliferation, ultimately leading to the selection of more B cells with acquired N-glycosylation sites within the variable regions of the BCR.

We speculate that our findings may also provide a strong rationale for the research and development of alternative therapies that seek to modulate autoimmune diseases through the use of anti-glycosylating compounds (13) or glycans that can competitively bind to lectins on dendritic cells and thereby interfere with the recruitment of a deleterious (auto)immune response (14).

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SAMENVATTING

Dit proefschrift richt zich op de bestudering van de genen die coderen voor de zware ketens van de immunoglobulines (IGHV) in auto-immuunziekten met speciale aandacht voor het Sjögren syndroom (pSS) in de eerste hoofdstukken. Door de studies over pSS patiënten waren wij in staat om nieuwe inzichten over auto-immuunziekten te krijgen. Daarna hebben wij deze strategie ook toegepast om andere auto-immuunziekten te bestuderen. Onze studies over IGHV gen sequenties die coderen voor een deel van de B cel receptor (BCR) toonden nieuwe en belangrijke inzichten aan over de selectie van B cellen in auto-immuunziekten.

Het belang van B cellen voor auto-immuunziekten zoals pSS, Reumatoïde Artritis (RA) en Systemische Lupus Erythrematosus (SLE) werd duidelijk toen therapie waarbij B cellen worden verwijderd succesvol waren in het verminderen van de klinische klachten bij behandelde patiënten (1). Naast de effectiviteit van deze therapie, suggereerde de gelijktijdige terugkeer van ziekte verschijnselen en de terugkeer van de B cellen ook dat bepaalde B cel populaties betrokken zijn bij de terugkeer van de ziekte. Dit was de basis van onze eerste studie (Hoofdstuk 3) over pSS patiënten die behandeld waren met Rituximab (RTX), een antistof therapie die B cellen depleteert (2). In deze studie gebruikten we de IGHV sequenties als klonale B cel merkers om de aanwezigheid van bepaalde klonale B cel populaties aan te tonen voor en na behandeling. Belangrijk in deze studie was dat we konden beschikken over biopsies van de parotis speekselklier, niet alleen voor aanvang van de therapie maar ook 12-16 weken en 36-52 weken na therapie. Het laatste tijdstip was een moment waarop de tekenen van terugkeer van de ziekte duidelijk aanwezig waren. Hierdoor waren wij in staat om bepaalde immunoglobuline producerende cellen aan te tonen voor RTX behandeling die aanwezig bleven in de parotis speekselklieren zelfs na B cel depletie met RTX. De aanwezigheid van cellen die verschillende isotypes tot expressie brengen binnen deze groepen van klonaal gerelateerde cellen, suggereerde lokale isotype switching in de speekselklieren van pSS patiënten. Gebaseerd op deze studie, suggereerden we dat persisterende immunoglobuline producerende cellen mogelijk bijdragen aan de terugkeer van de ziekte na behandeling (2).

Onze volgende stap was om de veranderingen die plaats vinden in het immunoglobuline repertoire in de parotis speekselklieren van de pSS patiënten ten gevolge van de RTX behandeling te beschrijven. Dit vormde de basis van hoofdstuk 4. Ten eerste hebben we bepaald wat de karakteristieke eigenschappen zijn van de immunoglobuline producerende cellen in de aangedane parotis speekselklieren van pSS patiënten door die eigenschappen te vergelijken met die van parotis speekselklieren van controle patiënten. In deze studie zagen we de volgende eigenschappen die significant verhoogd waren bij pSS patiënten (voor behandeling met RTX) in vergelijking met controle patiënten: (a) Verhoogde klonale expansie van immunoglobuline producerende cellen die IGHV-3 familie genen tot expressie brengen (b) Een hogere expressie van IgG1 in vergelijking met andere IgG subklassen (c) Meer conservering van de IgG framework regio's (FRWs) (d) Een verhoogde frequentie van verkregen N-glycosylering motieven in de variabele delen van de zware keten van de IgG immunoglobulines. B cel depletie met RTX veranderden deze afwijkende eigenschappen van de immunoglobuline-producerende cellen niet in behandelde pSS patiënten. Deze studies gaven meer aanwijzingen die onze eerdere conclusie ondersteunden dat immunoglobuline producerende cellen die persisteren na RTX kunnen bijdragen aan de terugkeer van de ziekte (2). We vermoedden ook dat de tijdelijke klinische verbetering na RTX waarschijnlijk veroorzaakt wordt door de verwijdering van B cellen en CD20+ plasma cellen (3) waardoor een lager niveau van autoantistoffen geproduceerd wordt door kort levende plasmacellen en/of door de verlaging van andere B cel effector functies, zoals antigen-presentatie en cytokine productie (4, 5). We suggereerden dat de onderliggende auto-

immuun mechanismen waarschijnlijk onderhouden worden door lang-levende plasmacellen zoals ook verondersteld in SLE (4, 6-8).

Voor zover wij weten was dit de eerste studie die een rol voor N-glycosylering bij de selectie van immunoglobuline producerende cellen in pSS patiënten liet zien. Wij veronderstelden dat een deel van de B cellen in pSS patiënten mogelijk geselecteerd worden ten gevolge van de verkregen N-glycosylerings motieven in de immunoglobuline variabele regio's. Hierdoor vroegen wij ons af of dit fenomeen mogelijk ook een rol zou kunnen spelen in andere auto-immuunziekten en dit leidde tot de studie beschreven in hoofdstuk 5. Deze studie laat duidelijk zien dater in patiënten met auto-immuunziekten significant meer B cellen worden geselecteerd met potentiële N-glycosylering motieven die verkregen zijn door somatische hypermutaties in vergelijking met de B cellen afkomstig uit niet-auto-immuun weefsels, maar ook in vergelijking met antigeen-specifieke repertoires. Deze selectie was zelfs nog duidelijker binnen de populaties van IgG producerende cellen. Op grond hiervan stelden we een nieuwe hypothese op dat de aanwezigheid van bepaalde verkregen N-glycosylerings motieven (ac-Nglycs) in IGHV sequenties in bepaalde auto-immuunziekten kan duiden op een afwijkende selectie druk die ervoor zorgt dat B cellen met geglycosyleerde BCR beter kunnen worden geactiveerd en overleven in bepaalde weefsels in patiënten met auto-immuunziekten door de interactie met lectin-producerende cellen zoals bijvoorbeeld dendritische cellen of macrofagen (9).

Het meest opvallendevan onze resultaten in hoofdstuk 5 was de observatie dateen grootdeel van de ac-Nglycs plaatsvinden op dezelfde posities in de FWRs als die waar B cel super antigenen zoals Staphylococcal protein A (SpA), pFv (endogenous human gut-associated sialoprotein) en HIV-1 envelop eiwit, gp 120 aan kunnen binden (10). Deze eigenschap van B cel super antigenen zorgt voor de beschikbaarheid van bindingsmogelijkheden die slechts minimaal beïnvloed worden door de onvoorspelbaarheid van genetische recombinatie of door de hypervariabiliteit die geïntroduceerd wordt door SHM die voornamelijk gericht is op de complementarity determining regio's (CDRs). De vergelijking met BCR super antigen interactie wijst op twee belangrijke punten: Ten eerste omdat binding van super antigenen aan deze posities leidt tot B cel activatie, differentiatie en immunoglobuline expressie, zou eenzelfde binding aan de BCR bij andere moleculen kunnen leiden tot vergelijkbare responsen (10). Ten tweede kan de nadruk op de conservering van de structuur van de FWRs buiten de klassieke antigen-bindende delen (CDRs) ook duiden op B cel selectie. Vervolgens hebben wij onderzocht of dergelijke vormen van B cel selectie gedetecteerd kunnen worden in auto-immuun repertoires. Wij hadden al gezien in Hoofdstuk 4 dat de IGHV sequenties afkomstig van de aangedane speekselklieren van pSS patiënten een verhoogde conservering van de FWR structuur in vergelijking met die in controle patiënten laten zien ondanks het feit dat alle sequenties veel mutaties bevatten. Dit leek veel op wat beschreven is bij BCR super antigeen interacties. [Hamza et al, submitted] Met onze studie in Hoofdstuk 6 waren wij in staat om te bevestigen dat de nadruk op de conservering van de FWR structuur ook een eigenschap was van het IGVH repertoire in andere auto-immuunziekten zoals RA, SLE, multiple sclerose, Chaga's disease, granulomatosis met polyangiitis (eerder bekend als Wegener's granulomatosus) en ankylosing spondylitis. Bovendien liet het IGHV repertoire in auto-immuunziekten een gebrek aan positieve selectie zien in conventionele antigeen-bindende delen (CDRs). Deze eigenschappen van het auto-immuun repertoire waren significant afwijkend ten opzichte van controle en klassieke antigeen-geselecteerde repertoires, terwijl het veel overeenkomsten vertoonde met super antigeen geselecteerde repertoires. Onze bevindingen suggereerden dat de veranderde selectiedruk van B cellen aan de basis kan staan van auto-immuunziekten waarbij het verkrijgen van N-glycosylerings motieven in BCR variabele

delen een extra selectie of activatie signaal kan geven door binding aan lectin-positieve cellen van het niet-specifieke immuun systeem (9). Deze bevindingen bevestigden onze hypothese dat veranderde B cel selectie druk in auto-immuunziekten een bijdrage kan leveren aan de pathologie bij auto-immuunziekten.

Als vervolg op onze observaties in hoofdstukken 5 en 6 , hebben wij onze aanwijzingen over veranderde B cel selectie druk in auto-immuunziekten gebruikt om een hypothetisch model op te stellen met een mogelijke link tussen super antigenen en de pathologie van auto-immuunziekten. In dit model stellen wij voor dat bepaalde super antigeen infecties een bijdrage kunnen leveren aan een eerste veranderde selectie van het B cel repertoire. In personen met bepaalde genetische eigenschappen die een verhoogde kans geven op auto-immuniteit, kunnen de super antigeen infecties leiden tot de selectie en activatie van B cellen met bepaalde eigenschappen zoals N-glycolysering van de Fab regio's. Zo'n soort mechanisme was al eerder aangetoond bij het vrouwelijke hormoon progesteron dat in staat is om de N-glycosylering te beïnvloeden door haar invloed op de expressie van oligosaccharyltransferase (11). Gezien het feit dat auto-immuunziekten verhoogd voorkomen bij vrouwen ten opzichte van mannen, is het mogelijk dat een bepaalde verhoogde B cel activatie gecombineerd met een hormonale onbalans of andere genetische factoren de basis kan vormen van een predispositie van voortdurende B cel differentiatie en proliferatie, die uiteindelijk kan leiden tot de selectie van meer B cellen met verkregen N-glycosylerings motieven in de variabele delen van de BCR (12).

We speculeren dat onze bevindingen ook mogelijk aanleiding zijn voor verder onderzoek naar de ontwikkeling van alternatieve therapiemogelijkheden die proberen om het verloop van auto-immuunziekten te beïnvloeden door het gebruik van anti-glysosylerende moleculen (13) of glycanen die competitief binden aan lectinen op dendritische cellen en daardoor kunnen ingrijpen op een mogelijk schadelijke (auto) immuun response (14).

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AUTHOR'S COMMENTS

Success is simple: Do what's right, the right way, at the right time.

- Arnold H. Glasgow, American psychologist and author

I thank God, whose mercy and blessings ensured my writing this penultimate chapter in a journey that seared my psyche in more ways than one.

All the PhD comics and satire in the world could not have prepared me for what was, diplomatically put, the most challenging experience of my life. And this despite my having gone through the trauma of child-birth three times, the shortest of which were 22 hours. But while labor pain is over in hours, PhD programs last a mind-numbing 4 years, if not more, with a uniquely repetitive pattern of problems and challenges.

You can have any number of surveys or theories about the problems PhD students face anywhere in the world. But it's striking how few of them actually have to do with scientific or technical issues (as reported by the The Postgraduate Research Experience Survey, 2007). In my opinion, these problems are really symptoms of an underlying decay within scientific communities worldwide, where crony clubs and ego worship have overtaken critical thinking and scholarship as primary research motivations. Most certainly, the scientific community the world over is in dire need of honest, a-political and intellectually superior individuals to replace the many cunning politicians, brazen opportunists and spineless bureaucrats who've clawed their way to scientific legitimacy. If nothing else, it would certainly save a lot of money. Perhaps it's time for a new 'Renaissance' or an 'Arab spring-style' awakening in today's scientific 'Winterlands' too.

As I get ready to celebrate my freedom from all of this (at least for a while), I'd like to share a few gems of advice distilled from the PhD experiences of many colleagues as well as my own. This is for all those foreign students who are in the beginning or middle of their PhD programs in the Netherlands. Those who are in the Terminal stage may read it too and I can almost imagine the wind from all their heads bobbing in agreement at what follows:

1) Get a copy of the book, 'The Undutchables'. This book is THE ultimate guide to "dealing with the Dutch, before they deal with you" (quote from Martijn de Rooij, author of 'The Dutch I Presume?'). It's also a guide to understanding a population as unique as the Dutch landscape. There are some truly admirable traits in the Dutch which I hope to export to my own native Indian and adopted Omani cultures. The Dutch really are an uncomplicated people, even when they are contradicting themselves; and reading about their culture helps avert many a mistaken conclusion.

2) There is a popular Dutch saying, "'*Doe maar gewoon, dan doe je al gek genoeg*' (meaning: just act normal, that's strange enough). The Dutch are obsessed with socialist equality and the 'polder' format where everything is mashed into oblivion. And this applies to people too in that, no one must stand out. Most Dutch people resent A-personalities and high-achievers, particularly if they are foreigners or 'foreign-looking'. So if you exhibit any sort of 'outstanding' abilities, you will get 'poldered' down. On the other hand, PhD students who are average (or below) or are incapable of conducting experiments on their own (wonder why they get a PhD too?) will be given an army of technicians and under-grad/graduate students to finish their projects. I would've thought, people should match their talents to their jobs, not vice versa. But the Dutch think vice versa.

   This unnatural equalizing will be a shock to accomplished international students from thriving, populous meritocracies, where competition is fierce and 'outstanding-ness' is

THE key winning strategy. If you wish to benefit from your professional interactions within Dutch academia, prepare to blend in by subduing your intellect, personality and all signs of economic prosperity. I have seen this tactic work incredibly well for some colleagues.

3) Never expect an extension on your PhD programs even if you are promised it. Do not trust anything you are told until you have it in writing. Broken promises, retracted statements, thwarted opportunities and delayed projects are as common as animal excrements on Dutch streets. The Dutch will arrive at meetings sharply on time, however unholy the hours. But they are depressingly casual with long-term projects. They may argue that they prefer quality over speed. But when your PhD contract ends and salary stops, while your projects are far from completion due to inferior collaborations, this attitude can quickly seem like sadism at your expense. Although, a generous extension is a possibility, this luxury is not necessarily extended to non-European PhD students. So, be smart with your time.

4) Never lose your temper, although you will often have enough reason to do so. I have rarely seen Dutch people lose their temper and this is a beautiful aspect of Dutch culture. To Dutch people, losing your temper means you are out of control, whereas, in many eastern cultures, expressing anger is a way of regaining control or establishing authority. I have to say I infinitely prefer the Dutch way in this matter.

5) Learn to speak basic Dutch. The Dutch openly appreciate it when foreigners attempt to converse in their language. It also makes for better social networking because the Dutch have perfected the art of isolating a person, even in a crowd. But don't learn too much because then you get complaints about your accent.

6) If you are a woman, beware of the Alpha female; there's bound to be one or two in most departments and they come in all possible forms; technicians, post-docs, clinicians, professors etc. The phenotype of the Alpha female is instantly recognizable. She will single out younger females (preferably foreigners) for bullying or ostracizing, demand interference in projects or core authorships on articles she does not deserve and create a circle of cooing cronies who let her think she's always right (although, these cronies would probably ditch her at the first scent of trouble). Dutch men will often laugh these actions away saying, "It's a woman thing....", as if this can ever be an explanation for ridiculously bad behaviour.
If an Alpha female sees you as easy prey and if she has any authority over your project, my advice to you is to quit. If this is impossible, then get ready for a messy and UGLY situation. As someone famously said, while wisely staying anonymous:

"Oh! the gladness of a woman when she is glad!; Oh! the sadness of a woman when she is sad!; But gladness of her gladness and the sadness of her sadness.....Are nothing to her badness -- .....When she is bad !"

7) Never reveal future plans, ambitions or goals. The more you reveal the more hurdles some people will gleefully roll into your way. In other cultures, having your future planned out is seen as being responsible and visionary. In the Netherlands, it's seen as being over-confident.

8) If you ever get the chance to suggest a book for your department, suggest the following, "Faculty Incivility: The Rise of the Academic Bully Culture and What to Do About It" by Prof. Darla Twale and Barbara De Luca. It makes for an interesting, if not horrifying read for all aspiring to join scientific academia.

9) There are some phrases that are commonly said to students who perform way better than expected in order to bully them into intellectual submission. I mention some of them here along with what people actually mean when they say them. If you hear these too many times, you could be heading for serious trouble in your PhD.

What is said	What it actually means
You have lack of focus	You are a creative and independent thinker
It's a miscommunication	Something went wrong, everyone knows it's not your fault ....but you will suffer for it
You're homesick	You have complained about being treated unfairly
You need to socialize more	You make other students and technicians feel insecure with your productivity
You are stubborn	You have not buckled under pressure and bullying

10) Get the book, "The Undutchables". Did I say that already?..... Then I'm saying it again.

"Suffering makes for a sharper pen than joy"

ACKNOWLEDGEMENTS

"O mankind! We created you from a male and a female and made you into nations and tribes (so) that you may know and honor each other (not that you should despise one another). Indeed the most honorable of you in the sight of God is the most righteous."
-Holy Quran (49:13)

I thank God, that He has let me hope for the day I would complete my thesis because there were innumerable instances, when dreams of quitting this PhD seemed tantalizingly real. I am determined that I should not forget to thank all those who helped me weather out this long tempest.

First my family.....

Being married at 18 years, a mother at 19, getting a Bachelors degree, having two more children, doing a Masters, followed by two very demanding jobs for 5 years and then a 4yr-PhD abroad..... all in that order, ensured that the past 16 years of marriage to me would be a tumultous rock 'n roll for my husband. Yet, through it all, he remains a steady source of love and comfort. Despite being a better scholar than I, he gave up his Masters degree to provide me a quiet shade of unyielding support for my career.

My parents too were not spared. While I went out to study or work, my Mum stepped in every morning to re-live the pangs of motherhood with my three sons, without complaint or regret. Instead, with every step of my growth, she pushed me to achieve more. She tirelessly devoted her life to give me everything she'd deserved and been denied because of her gender. And my Dad, despite being a very busy and highly-respected physician in society, shared in the late night vigils of colic and paediatric fevers, while I sat and studied for exams. Even my brother and sister-in-law have chipped in for their full quota of babysitting blues.

To my darling husband and loving parents,.... no words can describe the sacrifices you made to see me get this far. "Thank you" seems so very inadequate now. I have been infinitely blessed by God in that He gave you to me. It was your unflinching support and immeasurable love that helped me survive the ordeals of my PhD.

To my dearly beloved sons, ...Mohammed (15yr) ,Adam (12yr) and Dawud (10yr). I now truly understand why the Holy Quran refers to children as the 'coolness of the (parents') eyes'. My eyes have been sore these past years I spent away from you. I don't know if you will ever forgive me for the years I was missing from your life. Insha Allah, someday I hope you will understand why I did this, even if the world doesn't. The thought of returning without a PhD and wasting the time I'd already spent away from you, kept me going, when the going got tough.

To my dear brother, sister-in-law and your children: Maariya (12yr) – our clever princess, Abdullah (7yr) – the sensitive gentleman, Abdur-Rahman (6yr) – the adorable conqueror of hearts and sweet little Aliya (5 months),.... you make me feel so fortunate to be a sister and an aunt.

Now, to thank all those who supported me in my professional life.....

To Prof. Nicolaas Bos, my PhD supervisor, I am extremely grateful for your assistance during my PhD. Your enthusiasm for research is quite infectious and I must admit that I am awed at how you managed to juggle your many responsibilities in research and education together. I truly enjoyed our scientific discussions and good-natured sparring during manuscript revisions. I will always be grateful for your generous support, guidance and patience. THANK YOU.

To Prof. Cees Kallenberg, thank you for your gentle support throughout my PhD. I remember the times I sent you a manuscript for review and it would be sent back with comments in a day or two!....Even when you were at your busiest. This shows you value other people's time and efforts just as you value your own,.....a rare quality indeed! Your enthusiasm for research even after being officially retired, is a source of true inspiration. A gentlemen in every sense..., dear sir, it was a pleasure working with you. I will always remember you and your wife, Dineke, with much fondness.

To Prof. Frans Kroese, I am grateful to you for providing the facilities and assistance required to complete my experiments. I would also like to thank you for the considerable time you spent reviewing my manuscripts and for your exhaustive advice on scientific writing.

To Prof. Hendrika Bootsma, thank you for providing me with patient samples for my PhD project and for any other positive input, you may have given to my PhD projects.

To dear Dr. Anna Rajab; my boss and mentor during my job at the Ministry of Health, Oman; thank you very much for giving me my first big break. I learnt and accomplished so much while working for you. You gave me the honour of designing the labs for the upcoming National Genetic Center in Oman and in doing so, opened a world of opportunities I could never have entered on my own. The training scholarships to NHS,Oxford and LUMC, Leiden that you encouraged me to take up, shaped my interest in research. You also encouraged me to do a PhD and here I am. I owe you more gratitude than I can express and look forward to continuing our collaboration in the future.

To Prof. Arjan Visser, thank you for your consistent positivity. Even in the midst of embarrasingly rude situations that I faced undeservedly, I could always rely on your kind smiles and your quiet encouragement. I am also grateful for your amazingly prompt responses to my requests for article review.

To my co-PhD student Drs. Rodney Pollard, I am grateful to you for providing the clinical information on all the patients used in my experiments, which I'm afraid I would not have been privy to if it hadn't been for you. I have no doubt that your good nature and quiet disposition will be a valued addition wherever you go.

To Annie Visser, I am extremely grateful for the immense technical and logistical support you provided for helping me complete my experiments without major glitches. I also thank you for your warmth and friendship.

To Dr. Alja Stel, Minke Huitema and Johan Bijzet, thank you for your bright smiles and kind words at all times.

To Dr. Wayel Abdulahad, thank you for your collaboration in my project and your able advice.

To others on the Sjogren's team - Prof. Fred Spijkervet, Drs. Dan Zhang, Dr. Jiska Meijer and Drs. Petra Meiner, thank you for for your acquaintance and your participation in my projects.

Dear Greetje, Sippie and Janny, as secretaries of the departments where I worked, I am grateful to you for all the help and support you gave me these past four years. Especially Greetje who ensured that we, the eternally-harried PhD students, never ran out of coffee and tea. I sometimes think Greetje went through her own research project, trying to find out which tea brand was dark enough to satisfy my Indian taste. This could not have been easy, since we Indians cook our tea instead of brewing it. But sufficient to say, Greetje was indeed successful.

To Dr Davina Opstelten, Dr. Jeroen Visser, Dr. Sylvia Biejer-Liefers and Lisa Galzenburg, my colleagues at what was formerly a bustling Immunology section at the Cell Biology department, it was nice to have known and worked with you. I enjoyed spending the departmental lab-days and dinners with all of you. To Davina, in particular, thank you for your heartfelt and timely advice on many occasions. I have no doubt that should you so choose, you would make a more discerning and intuitive PhD supervisor than many others I've known. And to Jeroen, one of the kindest individuals I met at UMCG, I sincerely wish you success in your new research group.

To my co-PhD students working at or in conjunction with the Dept. of Rheumatology and Clinical immunology, Deena, Sarah, Paulina, Nato, Kasia, Alexander, Neils, Nynke and Suzanne, I wish you all success in your careers.

I sincerely thank Ms. Geri Hoogenberg, Prof. Han Moshage, Ms. Rieke Banus, Ms. Mathilde Pekelaar, and Ms. Bansema for their advice and help on several occasions.

I also thank Prof. Maikel Pepplenbosch, who now works at Erasmus MC, Rotterdam, for interviewing and recommending me for this PhD position.

I'd also like to thank my former colleagues, Jacobus, Rajesh, Kaushal, Saravanan and Gwenny for any help they gave me and wish them all the best for the future. I am especially grateful to Jacobus for his brave support and kindness when I once faced a traumatizingly discriminatory situation.

I also wish to thank Henk and Geert from the FACS facility for their help in my FACS experiments.

Now, out of the professional coop,.....

I was extremely fortunate in the friendships I forged during my time in the Netherlands, which reached out to me unconditionally in my times of happiness and distress. To all my dear friends..... *thank you.* The phrase, "friends for life" means so much more now, thanks to Facebook, Orkut and Twitter.

To Divya, my dearest friend, confidante and paranymph, I think I learnt more lab techniques from you than I did from any other individual. Our coffee & gossip sessions after work was a time I looked forward to everyday. The gifts of your friendship and wisdom are treasures that I will cherish forever. With your uniquely superb intellect, a research lab would have to be a lucky one to have you working for it.

To my dearest Hanke, my Dutch sister, I so love your humour and easy warmth. Everytime you walk into a gathering, its like a light switches on and everyone suddenly realizes that life is wonderful indeed. That's the positivity you bring into the people you meet. And that cute little boy of yours is surely going to give a lot of girls a thumping heartache when he grows up. May God make all our children among those who are righteous in this world and blessed in the next, Ameen. I look forward to the day when you and Hamza will visit me in Oman, Insha Allah.

To Deena, my dear friend, I enjoyed every moment I spent in your company. I am grateful to have had your friendship throughout our common adventure as 'PhD students from the Middle-east'.

To Heba, my dear Egyptian friend and former housemate, you love your friends with a passion that's probably only matched by your passion for the Egyptian revolution. You are the sister I always wanted and I am grateful to God for having known the warmth of your friendship and the beauty of your heart. If only more people were like you, no one would wish for Utopia.

To my dear friend, paranymph and co-PhD student Sarah, you are probably the youngest and quietest friend I have, but your silence hides a wisdom beyond your years. You have an uncanny gift for judging people and I can only wish that I were as astute as you in this. I am grateful and touched by your efforts to cheer me up during some very testing times.

To my dear Rohani, thank you for being the sweet and cheerful person you are. I'll always cherish the memories of the Ramadan we spent together. Ramadan is a month when families come together to mesh into a cosy spiritual bliss. It was comforting to break my long fasting days with you at a time when I ached for my family back home.

To my dear Heleen, thank you for your friendship and warmth. I haven't been able to spend much time with you, especially these last few months. I hope to make up for it by inviting you, Willem and your boys to visit and stay with me and my family in Oman, Insha Allah.

To Babbani and Gulbahar, thank you for hosting me in your house for the last 8 months and for cooking more-than-I-could-possibly-eat scrumptious Turkish dinners for me every day. It was a relief not to have to cook during the last hectic months of my PhD. May God reward you well for your kind and gracious hearts.

Dear Monireh, my colleague and former housemate, I will never forget the care you gave me for a full 3 weeks when I was unable to walk from a twisted ankle. I am indebted to you for the friendship you showered on me these past years.

To all my other Ladies Day Club sisters, Daisy, Habon, Yasmina, Jolien, Aletta, Satia, Faduma and Kirsten, it was wonderful knowing you all and I will miss being part of your circle.

To my friends Nasreen, Salli, Nada, Hassiba and Suad, I haven't spent as much time with you as I would have liked, since we were all quite busy with our studies and lives. Thank you for your warm friendships. I pray that you will all be successful in your lives, Ameen.

"Verily with every hardship there is relief."
- The Holy Quran (94:6)

LIST OF PUBLICATIONS

PUBLICATIONS AND PAPERS

- Pollard RP, Abdulahad WH, Vissink A, **Hamza N**, et al. Serum levels of BAFF, but not APRIL, are increased after rituximab treatment in patients with primary Sjogren's syndrome: data from a placebo-controlled clinical trial. AnnRheum Dis. 2012 Jul 31 [research article]
- **Hamza, N**; Bos, NA & Kallenberg, CGM. B cell populations and sub-populations in Sjögren's syndrome. La Presse Medicale, 2012 Jul 26 [review]
- **Hamza, N**; Bootsma, H; Yuvaraj, S; et al. Persistence of immunoglobulin-producing cells after Rituximab treatment in primary Sjögren's syndrome patients. Annals of the Rheumatic Diseases; 2012, in press [research article]
- **Hamza N**; Bootsma, H; Pollard, RPE; et al. Clonal Analysis of Ig-Expressing Cells in Salivary Glands of Primary Sjögren's Syndrome Patients after Anti-CD20 Treatment. Arthritis Rheum 2010;62 Suppl 10 :1889 [abstract].
- Hassan SM, **Hamza N**, Jaffer Al-Lawatiya F,et al. Extended molecular spectrum of beta- and alpha-thalassemia in Oman, Hemoglobin. 2010 Jan;34(2):127-34. [research article]

MANUSCRIPTS SUBMITTED OR IN PREPARATION

- **Hamza, N**; Hershberg, U; Kallenberg, CGM; et al. Immunoglobulin gene analysis reveals altered selective pressures in parotid glands of Sjögren's syndrome patients [research article]
- **Hamza, N**; Kroese, FGM & Bos, NA. Altered B cell selection in autoimmune disorders [research article]
- **Hamza, N**; Uduman M; Zhang, B; et al. Evidences for altered selection pressures on B cells in autoimmune disorders. [research article]
- **Hamza, N**; Kallenberg, CGM; Bos, NA. A hypothetical model for altered B cell selection in autoimmune diseases. [review]

Nishath Hamza was born on 18 November, 1977 in Kerala, India. She grew up in Muscat, Oman where her physician father, mother and elder brother have been living since 1975. She attended the Indian School in Muscat and finished her high school in 1995. She then spent a gap year in USA doing foundation courses in Chemistry and Physics at universities in Texas and New Jersey.

After her marriage in September 1996, Nishath decided to relocate to India and went on to complete a double major B.Sc in Chemistry and Environmental Management from the Mahatma Gandhi University, Kerala, India. After her father obtained Omani citizenship, Nishath enrolled at the Sultan Qaboos University in Oman and graduated with a Masters degree in Biochemistry and Molecular Biology in November 2004. In January 2005, she joined the Institute of Bioinformatics, Bangalore, India for a short stint as a Trainee Research Scientist. Then, from October 2005 to April 2008, she was employed with the Ministry of Health, Oman as a Molecular Geneticist under the supervision of Dr. Anna Rajab.

During this period, Nishath received scholarships funded by the Ministry of Health, Oman to train at the National Hemoglobinopathy Reference Laboratory, Oxford, UK and the Hemoglobinopathies Laboratory, Leiden University Medical Center, the Netherlands. As a result, she and her colleagues were successful in setting up the first National Molecular Genetic laboratory for diagnosis of Sickle-cell diseases and Thalessemias. Meanwhile, Nishath's natural inclination towards scientific pursuits prodded her towards a career in research and in May 2008, she joined the Department of Rheumatology and Clinical Immunology as a PhD student under the supervision of Prof. dr. Nicolaas A. Bos.

At present, Nishath lives with her husband, parents and three sons aged 15 yrs, 12 years and 10 years in Muscat, Oman.